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Additional inventors are being named on the ______ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Detection and Treatment of Fibrotic Disorders

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Docket No. : UF-418CP

Applicants : Nasser Chegini, Xiaoping Luo, Li Ding, R. Stan Williams

For : Detection and Treatment of Fibrotic Disorders

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DESCRIPTION

DETECTION AND TREATMENT OF FIBROTIC DISORDERS

The subject invention was made with government support under a research project supported by the National Institutes of Health Grant No. HD37432.

Background of Invention

Leiomyomas are benign uterine smooth muscle tumors, accounting for more than 30% of hysterectomies performed in the United States annually. Leiomyomas consist mainly of smooth muscle cells of myometrial origin and a network of connective tissue (Anderson, *Semin. Reprod. Endocrinol.*, 1996, 14:269-282; Chegini, *Cytokines and Reproduction,* 1999, 133-162).

Abnormal vaginal bleeding, pelvic pain and pelvic masses are among the major symptoms associated with leiomyomas. Leiomyomas are considered to originate from cellular transformation of myometrial smooth muscle cells and/or connective tissue fibroblasts during the reproductive years. The identity of factors that initiate such cellular transformation is not known; however, ovarian steroids are essential for leiomyoma growth, and GnRH anolog (GnRHa) therapy, creating a hypoestrogenic condition, is often used for their medical management (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Takeuchi, H *et al. J Obstet Gynaecol Res*, 2000, 26:325-331; Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32; Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223).

Hypoestrogenic conditions created by GnRHa therapy affect both leiomyoma and myometrium; however, clinical observations indicate a difference in their response to changes in the hormonal environment (Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223). In addition to GnRHa therapy, clinical and preclinical assessments of selective estrogen and

progesterone receptor modulators, either alone or in combination with GnRHa therapy, have shown efficacy in leiomyoma regression (Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32).

GnRHa-induced leiomyoma regression is accompanied by alterations in uterine arteriole size, blood flow, and cellular content as well as changes in the expression of several growth factors, cytokines, extracellular matrix, proteases, and protease inhibitors (reviewed in Chegini, *Cytokines in Human Reproduction,* 2000, 133-162; Nowak, *Bailliere Best Pract Res. Clin Obstet. Gynaecol.,* 1999, 13:223-238). Differential expression and autocrine/paracrine action of many of these molecules are considered to play a central role in leiomyoma growth and GnRHa-induced regression (Chegini, *Cytokines in Human Reproduction,* 2000, 133-162; Nowak, *Bailliere Best Pract Res. Clin Obstet. Gynaecol.,* 1999, 13:223-238).

At the cellular level, a combination of mitotic activity, cellular hypertrophy, and accumulation of extracellular matrix (ECM) are considered to participate in leiomyoma growth (Anderson, *Semin. Reprod. Endocrinol.,* 1996, 14:269-282; Chegini, *Cytokines and Reproduction,* 1999, 133-162; Stewart *et al., J. Clin. Endocrinal Metab.,* 1994, 79:900-906; Wolanska *et al., Mol Cell Biochem.,* 1998, 189:145-152). Compared to myometrium, leiomyomas are reported to overexpress estrogen and progesterone receptors, and GnRHa therapy lowers their content in both tissues (Stewart *et al., Semin, Reprod. Endocrinol.,* 1995, 10:344-357; Englund *et al., J. Clin. Endocrinol Metab.,* 1998, 83:4092-4092). Clinical and basic science research shows that GnRHa acting through suppression of the pituitary–gonadal axis cause leiomyoma to regress by affecting uterine arteriole size, blood flow at the tumor level. But its effect at cellular and molecular levels in leiomyoma has not been investigated.

With respect to the leiomyoma molecular environment, several genome-wide allel-typing studies have evaluated the association between genomic instability and the pathogenesis of leiomyoma (for review; Ligon, AH and Morton, CC *Hum Reprod Update*, 2001, 7:8-14). These studies have led to the identification of several candidate genes, however in the majority of cases evidence of genomic instability is either lacking or inconsistent (Ligon, AH and Morton, CC *Hum Reprod Update*, 2001, 7:8-14), implying the existence of unrecognized pathways that can lead to the development of leiomyoma. Further studies have provided support for various

autocrine/paracrine regulators in the pathogenesis of leiomyoma including local estrogen production, growth factors, cytokines, chemokines and their receptors, whose expression are regulated by ovarian steroids (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20). These studies in many instances demonstrated altered expression of these factors and/or their receptors in leiomyoma compared to normal myometrium. In recent years cDNA microarray has been utilized as a high throughput method to identify a large number of differentially expressed and regulated genes in various tissues and cells. Using this approach, several recent studies have further assisted in fingerprinting the gene expression profile of leiomyoma and myometrium during the menstrual cycle (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). However, only the expression of a few of these newly identified genes has been validated, and their regulation and correlation with pathogenesis of leiomyoma remains to be investigated.

With respect to GnRHa therapeutic action, it is traditionally believed to act primarily at the level of the pituitary-gonadal axis, and by suppressing ovarian steroid production causes leiomyoma regression. However, the identification of GnRH and GnRH receptor expression in several peripheral tissues, including the uterus, has implicated an autocrine/paracrine role for GnRH and additional sites of action for GnRHa therapy (Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8). Demonstration of the expression of GnRH, as well as GnRH I and II receptors mRNA in leiomyoma and myometrium and their isolated smooth muscle cells has provided support for this concept (Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)). Several *in vitro* studies have also demonstrated GnRHa direct action on various cell types derived from

peripheral tissues resulting in alteration of cell growth, apoptosis, the expression of cell cycle proteins, growth factors, pro- and anti-inflammatory cytokines, proteases, and protease inhibitors (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Wu, X *et al. Acta Obstet Gynecol Scand*, 2001, 80:497-504). Local expression and differential regulation of these genes influences cell proliferation, differentiation, migration, inflammatory response, angiogenesis, expression of adhesion molecules, ECM turnover and apoptosis, *etc*., processes that are central to leiomyoma growth and regression (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Wu, X *et al. Acta Obstet Gynecol Scand*, 2001, 80:497-504; Dou, Q *et al. Mol Hum Reprod*, 1997, 3:1005-1014; Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-4143; Senturk, LM *et al. Am J Obstet Gynecol*, 2001, 184:559-566; Sozen, I *et al. Fertil Steril*, 1998, 69:1095-1102; Gustavsson, I *et al. Mol Hum Reprod*, 2000, 6:55-59; Orii, A *et al. J Clin Endocrinol Metab*, 2002, 87:3754-9; Fukuhara, K *et al. J Clin Endocrinol Metab*, 2002, 87:1729-36; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50; Ma, C and Chegini, N *Mol Hum Repord*, 1999, 5:950-954). Microarray studies, including a small-scaled array, have also identified the expression profile of additional genes targeted by GnRHa in murine gonadotrope tumor cell line LβT2, human breast tumor cell line MCF-7 and

leiomyoma and myometrium (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Ma, C and Chegini, N *Mol Hum Repord*, 1999, 5:950-954; Kakar, SS *et al. Gene*, 2003, 308:67-77).

Transforming growth factors beta (TGF-β) is a multifunctional cytokine and key regulator of cell growth and differentiation, inflammation, apoptosis and tissue remodeling (Blobe, GC *et al. N Engl J Med*, 2000, 342:1350-1358; Flanders, KC *Int J Exp Pathol*, 2004, 85:47-64; Schnaper, HW *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-252; Clancy, RM and Buyon, JP *J Leukoc Biol*, 2003, 74:959-960; Olman, MA and Matthay, MA *Am J Physiol Lung Cell Mol Physiol*, 2003, 285:L522-6). While under normal physiological conditions the expression and autocrine/paracrine actions of TGF-β are highly regulated, alteration in TGF-β and TGF-β receptor expression and their signaling mechanisms often resulte in various pathological disorders, including fibrosis (Blobe, GC *et al. N Engl J Med*, 2000, 342:1350-1358; Flanders, KC *Int J Exp Pathol*, 2004, 85:47-64; Schnaper, HW *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-252; Clancy, RM and Buyon, JP *J Leukoc Biol*, 2003, 74:959-960; Olman, MA and Matthay, MA *Am J Physiol Lung Cell Mol Physiol*, 2003, 285:L522-6). Altered expression of TGF-β isoforms (TGF-β1, β2 and β3) and TGF-β receptors (type I, II and III) in leiomyoma and their isolated smooth muscle cells (LSMC) compared to normal myometrium has been observed (Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16). Recently, it has also been demonstrated that leiomyoma and LSMC express elevated levels of Smads, components of the TGF-β receptor signaling pathway, compared to myometrium and MSMC (Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361). TGF-β regulates its own expression and the expression of Smad in LSMC and MSMC, and through downstream signaling from this and MAPK pathways regulates the expression of c-fos, c- jun, fibronectin, type I collagen and plasminogen activator inhibitor 1 in these cells (Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)). Additionally, data have demonstrated the ability of TGF-β to regulate LSMC and MSMC cell growth (Tang, XM *et al. Mol Hum Reprod*, 1997, 3:233-40; Arici, A and Sozen, I *Am J Obstet Gynecol*, 2003, 188:76-83;

Lee, BS and Nowak, RA *J Clin Endocrinol Metab*, 2001, 86:913-920; Arici, A and Sozen, I *Fertil Steril*, 2000, 73:1006-1011).

Because leiomyoma growth is dependent on ovarian steroids, GnRHa therapy and most recently selective estrogen and progesterone receptors modulators are used for their medical management (Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32). It has been demonstrated that GnRHa therapy results in a marked down-regulation of TGF-β isoforms and TGF-β receptors expression and alters the expression and activation of Smads in leiomyoma as well as LSMC (Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16). It has also been shown that TGF-β expression in LSMC and MSMC is inversely regulated by ovarian steroid compared to their antagonists, ICI-182780, ZK98299, and RU486 (Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078). In addition, it has been shown that other cytokines such as GM-CSF, IL-13 and IL-15, which promotes myofibroblast transition, granulation tissue formation and inflammatory response, respectively, may mediate their action either directly or through induction of TGF-β expression in LSMC and MSMC (Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Ding, L *et al. J Soc Gyncol Invest*, 2004, 00, 00). From these observations, it was proposed that the TGF-β system serves as a major autocrine/paracrine regulator of fibrosis in leiomyoma (Dou, Q *et al. J Clin Endocrinol Metab*, 1996, Chegini, N *et al. J Clin Endocrinol Metab*, 1999; 81:3222-3230; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Tang, XM *et al. Mol Hum Reprod*, 1997, 3:233-40). Evidence has been developed reflecting the molecular environments directed by GnRHa therapy in leiomyoma and myometrium, as well as by GnRHa direct action in LSMC and MSMC (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Luo, X *et al.* (Accompanying manuscript)).

Brief Summary of Invention

The present invention provides a method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject (such as endometrium, peritoneal fluid, and/or smooth muscle cells); (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest as compared to normal tissue (such as myometrium); and (c) correlating the expression of the gene(s) with the presence or absence of the fibrotic disorder in the subject. Examples of reproductive tract disorders include, but are not limited to, leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, and other tissue fibroses. Fibrosis involves the deposition of large amounts of extracellular matrix molecules, notably collagen. Fibrosis is involved in normal physiological responses (*e.g.*, wound healing) as well as pathophysiological conditions such as renal failure, liver cirrhosis and heart disease. The compositions and methods of the present invention are useful for detecting or treating abnormal fibrotic changes in the tissue of a subject.

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated

3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abi-interactor 2 (Abi-2); and other differentially expressed genes disclosed herein.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

The step of analyzing expression of the differentially expressed gene can be performed by quantifying the amount of differentially expressed gene product present in the sample, *e.g.*, by contacting the sample with an antibody that specifically binds the gene product. This step can also be performed by quantifying the amount of a nucleic acid that encodes the gene product present in the sample, *e.g.*, by contacting the sample with a polynucleotide that hybridizes under stringent conditions to the nucleic acid that encodes the gene product. The latter can also be performed using a polymerase chain reaction (PCR), for example.

Preferably, expression of a plurality of differentially expressed genes is analyzed. In this case, step (c) of correlating the expression of the differentially expressed gene with the presence or absence of the fibrotic disorder in the subject can include determining the ratio of two or more differentially expressed gene products in the sample.

In another aspect, the invention features a method for modulating gene expression in fibrotic tissue. This method includes contacting the tissue with an agent that modulates expression of a differentially expressed gene in the tissue. The fibrotic tissue can be from a subject with leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, or other tissue fibroses, for example. The agent can be one that specifically binds the product that is expressed by a differentially expressed gene. The agent can also be a nucleic acid that modulates (*i.e.*, increases or decreases) expression of one or more differentially expressed genes in a cell. The agent can also be one that modulates transcription or translation of a nucleic acid encoding the product of one or more differentially expressed genes. Thus, the agent can take the form of a polynucleotide, such as an antisense oligonucleotide. In other variations of this method, the agent can be an ovarian steroid, such as estradiol and medroxyprogesterone actetate. However, the agent is preferably not a hormone, but is nonetheless capable of modulating the expression of one or more genes that is differentially expressed in a fibrotic disorder, such as those genes differentially expressed upon GnRHa therapy.

<u>Brief Description of Drawings</u>

Figure 1 shows hierarchical clustering analysis of differentially expressed genes in leiomyoma and matched myometrium form untreated (f and m 315, 316 and317) and GnRHa

treated (f and m 287, 312 and 314) groups identified following unsupervised and supervised analysis in R programming environment and ANOVA with false discovery rate of $p \leq 0.02$ (22-25). Each column represents data from a single cohort with shades of red and green indicating up- or down-regulation of a given gene according to the color scheme shown below. Genes represented by rows were clustered according to their similarities in expression patterns for each tissue and treatment. The dendrogram displaying similarity of gene expression among the cohorts is shown on top of the overview image, and relatedness of the arrays is denoted by distance to the node linking the arrarys. The gene-tree shown at the left of the image corresponds to the degree of similarity (Pearson correlation) of the pattern of expression for genes across the experiments. The clustering divided the genes into five clusters designated as A to E and their zoomed images are presented in figure A to E. Genes that appear more than once are represented by multiple clones on arrays.

Figure 2 shows K-means clustering of genes regulated in leiomyoma and matched myometrium during growth and under the influence of GnRH therapy. The 390 differentially expressed gene values identified in these cohorts described in Figure 1, were subjected to k-means clustering grouping the genes into clusters based on similarity of expression in GnRHa treated and untreated cohorts. The analysis grouped the genes into 5 clusters (A-E). The rows represent the genes and columns the samples (f and m) from GnRHa treated (287, 312 and 314) and untreated (315, 316, 317) cohorts with shades of red and green indicating up-regulation or down-regulation of a given gene that are clustered according to their similarities in expression patterns.

Figure 3 shows gene ontology assessment and division of the differentially expressed genes in leiomyoma and myometrium and in response to GnRH therapy into similar functional categories illustrated as bar graphs with the percentage of total number of gene in each group showing in the front of each bar. Figures A-D show gene ontology assessment in untreated leiomyoma vs myometrium (A), GnRH-treated leiomyoma vs myometrium (B), GnRHa-treated vs untreated leiomyoma (C) and GnRHa-treated vs untreated myometrium (D).

Figure 4 shows hierarchical clustering analysis of 281 differentially expressed and regulated genes in LSMC (f) and MSMC (m) in response to GnRHa (0.01 μM) treatment for 2, 6

and 12 hrs or untreated control (C). The genes were identified following unsupervised and supervised analysis of the expression values and statistical analysis in R programming environment and ANOVA with false discovery rate selected at $p \leq 0.005$ (22-25). Each column represents data from a single treatment with shades of red and green indicating up- or down-regulation of a given gene. Genes represented by rows were clustered according to their similarities in expression patterns for each cell type and treatment. The dendrogram displaying similarity of gene expression among the cohorts is shown on top of the overview image with array relatedness is denoted by distance to the node linking the arrarys. The gene tree shown at the left of the image corresponds to the degree of similarity (Pearson correlation) of the pattern of expression for genes across the experiments. The clustering divided the genes into four clusters designated as A to D and their zoomed images are presented in figure A to D. Genes that appear more than once are represented by multiple clones on arrays.

Figure 5 shows K-means clustering of 281 differentially expressed and regulated genes in response to time-dependent action of GnRHa in LSMC and MSMC. The gene values identified in these cohorts as described in figure 1, were subjected to k-means clustering grouping the genes into clusters based on similarity of expression in response to time-dependent action of GnRHa. The analysis grouped the genes into 6 clusters (A-F). The rows represent the genes and columns the samples (f and m) and treatments with GnRHa for 2, 6 and 12 hrs and untreated control (C) with shades of red and green indicating up-regulation or down-regulation of a given gene that are clustered according to their similarities in expression patterns. Line graphs displaying the Standard division from the mean for each cluster in MSMC and LSMC in response to GnRHa time-dependent action for 2, 6 and 12 hrs compared to untreated control (Ctrl).

Figure 6 shows gene ontology assessment and division of the differentially expressed and regulated genes in LSMC and MSMC in response to GnRH treatment into similar functional categories illustrated as bar graphs with the percentage of total number of gene in each group showing in the front of each bar.

Figure 7 shows the expression profile of a selected group of genes representing growth factors/cytokines/polypeptide hormones/receptors (first row), intracellular signal transduction

pathways (second row), transcription factors (third row), cell cycle (fourth row) and cell adhesion/ ECM/cytoskeletons (fifth row) in response to time-dependent action of GnRHa in LSMC and MSMC. Values on the x-axis represent an arbitrary unit derived from the mean gene expression value for each factor after supervised analysis, statistical analysis in R programming environment and ANOVA as described in Figure 4, with gene expression values for the untreated controls (Ctrl) set at 1.

Figure 8 shows comparative analysis of the expression profile of 10 genes identified as described in Figure 1 as differentially expressed in response to GnRH therapy in leiomyoma and matched myometrium and untreated group by microarray and Realtime PCR. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene with values for the untreated controls (Crtl) set at 1. Total RNA isolated from these tissues was used for both microarray analysis and Realtime PCR validating the expression of IL-11, EGR3, CITED2, Nur77, TEIG, TGIF, p27, p57, Gas1 and GPRK5. On the Y-axis untreated myometrium and leiomyoma are designated as Unt-MM and Un-LM, and GnRH- treated as GnRH-Trt MM and GnRH-Trt LM.

Figure 9 shows comparative analysis of the expression profile of 10 genes identified as described in Figure 4 as differentially expressed and regulated in response to GnRHa time-dependent action in LSMC and MSMC by microarray and Realtime PCR. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene, and y-axis represent the time course of GnRHa (0.1μM) treatment (2, 6 and 12 hrs) with untreated control (Crtl) gene expression values set at 1. Total RNA isolated from these cells used for both microarray analysis and Realtime PCR for validating the expression of IL-11, EGR3, TEIG, TGIF, CITED2, Nur77, CDKN1B (p27), CDKN1C (p57), Gas1 and GPRK5.

Figure 10 shows results of Western blotting of IL-11, P27, P57, TIEG and TGIF illustrating the presence of their immunoreactive proteins in leiomyoma and myometrium from the untreated cohorts.

Figure 11 shows immunohistochemical localization of IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 in leiomyoma and myometrium. Note the presence of immunoreactive IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 in association

with leiomyoma and myometrial smooth muscle cells, and cellular components of connective tissue and vasculature. Both nuclear (EGR3, Nur77, p27, p57) and cytoplasmic (IL-11) staining is observed. Incubation of tissue sections with non-immune mouse (A), rabbit (B) and goat (figure not shown) IgGs instead of primary antibodies during immunostaining served as controls (Ctrl) reduced the staining intensity. Mag: X150 and X300.

Figure 12 shows hierarchical clustering analysis of 310 differentially expressed and regulated genes in LSMC (f) and MSMC (m) in response to TGF-β1 (2.5 ng/ml) treatment for 2, 6 and 12 hrs or untreated control (C). The genes were identified following unsupervised and supervised analysis of the expression values and statistical analysis in R programming environment and ANOVA with false discovery rate selected at p $\leq$0.001. Each column represent data from a single time point using two independent cell cultures with shades of red and green indicating up- or down-regulation of a given gene according to the color scheme shown below. Genes represented by rows were clustered according to their similarities in expression patterns for each treatment and cell type. The dendrogram showing similarity of gene expression among the treatments/cells is shown on top of the overview image and relatedness of the arrays is denoted by the distance to the node linking the arrarys. The gene tree shown at the left of the image corresponds to the degree of similarity (Pearson correlation) of the pattern of expression for genes across the experiments. The clustering depicts five groups of genes designated as A to E and their zoomed images are presented in figure A to E. Genes that appear more than once are represented by multiple clones on arrays.

Figure 13 shows K-means clustering analysis of 310 differentially expressed and regulated genes in LSMC (f) and MSMC (m) in response to time-dependent action of TGF-β described in Figure 12. The gene expression values in these cohorts were combined and subjected to k-means clustering that grouped the genes into five clusters (A to E) based on similarity of expression over the three time-point and untreated control. The rows represent the genes and columns the samples with shades of red and green indicating up- or down-regulation of a given gene that are clustered according to their similarities in expression patterns. The line graphs display the Standard division from the mean (x-axis) for each cluster in MSMC and

LSMC in response to TGF-β time-dependent action for 2, 6 and 12 hrs (y-axis) compared to untreated control (Ctrl).

Figure 14 shows gene ontology assessment and division of the differentially expressed and regulated genes in LSMC and MSMC in response to TGF-β treatment into similar functional categories illustrated as bar graphs with the percentage of total number of gene in each group showing in the front of each bar. The figures A and B show gene ontology assessment for LSMC and MSMC treated with (A) TGF-β and (B) pretreatment with TGF-β type II receptor antisense for 24 hrs followed by TGF-β treatment as indicated in materials and methods.

Figure 15 shows the expression profile of a group of genes representing growth factors/cytokines/polypeptide hormones/receptors (first row), intracellular signal transduction pathways (second row), transcription factors (third row), cell cycle (forth row) and cell adhesion/ ECM/cytoskeletons (fifth row) in response to time-dependent action of TGF-β in LSMC and MSMC. Values on the x-axis represent an arbitrary unit derived from the mean gene expression value for each factor after supervised analysis, statistical analysis in R programming environment and ANOVA as described in figure 12, with gene expression values for the untreated controls (Ctrl) set at 1.

Figure 16 shows hierarchical clustering analysis of gene expression values in untreated and TGF-b-treated LSMC and MSMC pretreated with TGF-b type II receptor (TGF-b type IIR) antisense or sense oligomers. The cells were cultured in serum-free phenol-red free media for 24 hrs washed and treated with TGF-b type IIR antisense or sense oligomers for additional 24 hrs. The cells were then washed and treated with TGF-b (2.5 ng/ml) for 2 hrs with untreated cells serving as controls. Supervised analysis of the gene expression values and statistical analysis in R programming and ANOVA identified 54 genes at a false-discovery rate of rate of ≤ 0.001, with expression levels discriminated among the treatment groups and untreated control. Each column represent data from a single time point using two independent cell cultures (f314 and f316 for LSMC and m314 and m316 for MSMC) with shades of red and green indicating up- or down-regulation of a given gene according to the color scheme shown below. Genes represented by rows were clustered according to their similarities in expression patterns for each treatment and cell type. The dendrogram showing similarity of gene expression among the treatments/cells is

shown on top of the overview image and relatedness of the arrays denoted by the distance to the node linking the arrarys. The gene tree shown at the left of the image corresponds to the degree of similarity (Pearson correlation) of the pattern of expression for genes across the experiments. The clustering depicts three groups of genes (A to C) and their zoomed images are presented in figure A, B and C. Genes appearing more than once are represented by multiple clones on arrays.

Figure 17 shows hierarchical clustering analysis of gene expression in isolated LSMC and MSMC pretreated with TGF-b type II receptor (TGF-b type IIR) antisense for 24 hrs followed by TGF-b treatment for 2 hrs (f 314, f316, m314 and m316-antisense), GnRHa-treated cells for 2 hr (f-314G, f316G, m314G and m316G) and untreated control (C). Supervised analysis of the gene expression values and statistical analysis in R programming and ANOVA identified 222 genes with a false-discovery rate of rate of $p \leq 0.001$, whose expression levels discriminated among the treatment groups and untreated control. The clustering depicts four groups of genes (A to D) and their zoomed images are presented in figure A, B, C and D. Genes appearing more than once are represented by multiple clones on arrays.

Figure 18 shows comparative analysis of the expression profile of 12 genes identified as described in Figure 12 as differentially expressed and regulated in response to time-dependent action of TGF-β1 in LSMC and matched MSMC by microarray and Realtime PCR. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene and y-axis represent the time course of TGF-β (2.5 ng/ml) treatment (2, 6 and 12 hrs) with untreated control (Crtl) gene expression values set at 1. Total RNA isolated from these cells was used for both microarray analysis and Realtime PCR validating the expression of IL-11, EGR3, CITED2, Nur77, TEIG, TGIF, Runx1, Runx2, p27, p57, Gas1 and GPRK5.

Figure 19 shows a comparative analysis of the expression profile of Runx1 and Runx2 genes in leiomyoma (LM) and matched myometrium (MM) from untreated (un-Trt) and women who received GnRHa therapy (GnRHa-Trt) as well as in leiomyoma and myometrial smooth muscle cells (LSMC and MSMC) in response to GnRHa (0.1 μM) time dependent action (2, 6 and 12 hrs) as described in detail in accompanying manuscript (Ref#22) and in response to time-dependent (2, 6 and 12 hrs) action of TGF-β1 (2.5 ng/ml) determined by Realtime PCR. In microarray analysis Runx2 expression was not included since its expression value did not reach

the study standard. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene and y-axis represents the time course of TGF-beta and GnRHa treatments, with untreated control (Crtl) gene expression values set at 1. Total RNA isolated from these cells was used for both microarray analysis and Realtime PCR validation.

Figure 20 shows a bar graph demonstrating differential expression of fibromodulin.

Figure 21 shows a bar graph demonstrating differential expression of Abi-2.

Detailed Disclosure

The study disclosed herein was designed to further define the molecular environments of leiomyoma and matched myometrium during the early-mid luteal phase of the menstrual cycle, which is characterized by elevated production of ovarian steroids, compared with tissues obtained from hormonally suppressed patients on GnRHa therapy. The present inventors further evaluated the direct action of GnRHa on global gene expression and their regulation in leiomyoma and myometrial cells isolated from the untreated tissue cohort. These approaches enabled the identification of expression profiles of genes targeted by GnRHa. The present inventors validated the expression of 10 of these genes in these cohorts, and concluded that local expression and activation of these genes may represent features differentiating leiomyoma and myometrial molecular environments during growth as well as GnRHa-induced regression.

Microarrays have been shown to be of great value in understanding the molecular biology of many diseases, and they have been successfully used to classify various tumors based on their clinical phenotype or genetic background. In this experiment, the present inventors have used gene expression profiling to define the biological relationship between TGF-β and GnRH in tumor growth and regression, and try to unveil the complexity of leiomyoma genesis and development. The present inventors have evaluated the underlying differences between molecular responses directed by TGF-β autocrine/paracrine actions in LSMC and MSMC, and following interference with these actions using TGF-β receptor type II antisense oligomers treatment. Since TGF-β receptors expression is targeted by GnRHa in leiomyoma and myometrium, the present inventors further evaluated the gene expression profiles in response to TGF-β type II receptor antisense treatment and GnRHa-treated LSMC and MSMC to identify the

genes whose expression are the specific target of these treatments. Using this approach, several differentially expressed and regulated genes targeted by TGF-β autocrine/paracrine action were evaluated, and the expression of 12 genes in LSMC and MSMC in response to the time-dependent action of TGF-β was validated using Realtime PCR.

Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and Current Protocols in Molecular Biology, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Various techniques using polymerase chain reaction (PCR) are described, *e.g.*, in Innis *et al.*, PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859-1862, 1981, and Matteucci *et al.*, J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers. Immunological methods (*e.g.*, preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, *e.g.*, in Current Protocols in Immunology, ed. Coligan *et al.*, John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff *et al.*, John Wiley & Sons, New York, 1992. Conventional methods of gene transfer and gene therapy can also be adapted for use in the present invention. See, *e.g.*, Gene Therapy: Principles and Applications, ed. T. Blackenstein, Springer Verlag, 1999; Gene Therapy Protocols (Methods in Molecular Medicine), ed. P. D. Robbins, Humana Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C. P. Hodgson, Springer Verlag, 1996.

The following publications are specifically incorporated herein by reference in their entirety, including all figures, tables, and sequences, to the extent they are not inconsistent with the explicit teachings of this specification: U.S. patent publication US 2003/0032044 (Chegini *et al.*), filed July 17, 2002; international publication WO 03/007685 (Chegini *et al.*), filed July 17, 2002; international publication WO 00/20642 (Chegini *et al.*), filed October 1, 1999; U.S. patent

publication US 2003/0077589 (Hess-Stumpp *et al.*), filed September 25, 2001; and U.S. patent publication US 2001/0002393 (Palmer *et al.*), filed December 20, 2000.

I. Detecting Fibrotic Disorders

The invention provides a method for detecting a fibrotic disorder in the tissue of a subject. This method includes the steps of: (a) providing a biological sample obtained (*i.e.*, derived) from the subject (such as endometrium or peritoneal fluid); (b) analyzing the expression of a differentially expressed gene in the sample; and (c) correlating the expression of the differentially expressed gene with the presence or absence of the fibrotic disorder in the subject.

Examples of reproductive tract disorders include, but are not limited to, leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, and other tissue fibroses (*e.g.*, fibroids) (Smits G. *et al.*, N. Engl. J. Med., 2003, 349(8):760-766; Elchalal U. *et al.*, Human Reproduction, 1997, 12(6):1129-1137; Stewart E. *et al.*, Human Reproduction Update, 1996, 2(4):295-306; Shozu M. *et al.*, The Journal of Clinical Endocrinology & Metabolism, 86(11):5405-5411; Estaban J. *et al.*, Arch. Pathol. Lab. Med., 1999, 123:960-962; Lee W. *et al.*, The Korean Journal of Pathology, 2003, 37:71-73; and Kurioka H. *et al.*, Human Reproduction, 1998, 13(5):1357-1360).

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1;

annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abl-interactor 2 (Abi-2); and other

differentially expressed genes disclosed herein. The number of differentially expressed genes analyzed in the sample can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abl-interactor 2 (Abi-2).

Suitable subjects for use in the invention can be any human or non-human animal. For example, the subject can be a female animal, such as mammal, like a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, rat, or mouse. Because the experiments presented herein relate to human subjects, a preferred subject for the methods of the invention is a human, such as a human female. Particularly preferred are female subjects suspected of having or at risk for developing a fibrotic disorder within the reproductive tract, *e.g.*, a woman suspected of having or at risk for developing leiomyoma, endometriosis, or peritoneal adhesions based on clinical findings or other diagnostic test results.

The step of providing a biological sample obtained from the subject can be performed by conventional medical techniques. For example, an endometrial tissue sample can be taken from the subject by biopsy. As another example, a sample of peritoneal fluid can be taken from a subject by conventional techniques. Suitable methods are described in more detail in the Examples sections presented below.

The step of analyzing the expression of a differentially expressed gene in the sample can be performed in a variety of different ways. Numerous suitable techniques are known for analyzing gene expression. For example, gene expression can be determined directly by assessing protein expression of cells or fluid of a biological sample (*e.g.*, endometrial tissue or peritoneal fluid). Proteins can be detected using immunological techniques, *e.g.*, using antibodies that specifically bind the protein in assays such as immunofluorescence or immunohistochemical staining and analysis, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoblotting (*e.g.*, Western blotting), and like techniques. Expression of differentially expressed genes can also be determined by directly or indirectly measuring the amount of mRNA encoding protein in a cellular sample using known techniques such as Northern blotting and PCR-based methods such as competitive quantitative reverse

transcriptase PCR (Q-RT-PCR). Suitable methods for analyzing expression of differentially expressed genes are described below; nonetheless, other suitable methods might also be employed.

The step of correlating the expression of the gene with the presence or absence of the fibrotic disorder in the subject involves comparing the level of gene expression in the test biological sample with levels of gene expression in control samples, *e.g.*, those derived from subjects known to have or not to have the particular disorder. Thus, after quantifying gene expression in a biological sample from a test subject, the test result is compared to levels of gene expression determined from (a) a panel of cells or tissues derived from subjects (preferably matched to the test subject by age, species, strain or ethnicity, and/or other medically relevant criteria) known to have a particular disorder and (b) a panel of cells or tissues derived from subjects (preferably also matched as above) known not to have a particular disorder. If the test result is closer to the levels (*e.g.*, mean or arithmetic average) from the panel of cells or tissues derived from subjects known to have a particular disorder, then the test result correlates with the test subject having the particular disorder. On the other hand, if the test result is closer to the levels (*e.g.*, mean or arithmetic average) from the panel of cells or tissues derived from subjects known not to have a particular disorder, then the test result correlates with the test subject not having the particular disorder. Optionally, the method further comprises selecting and administering a therapy or therapies to the patient to treat for the correlated disorder(s).

II. <u>Modulating Gene Expression</u>

The present invention also provides a method for modulating the expression of genes that are differentially expressed in fibrotic tissues, compared to normal tissues. Restoration of gene expression to levels associated with normal tissue is expected to ameliorate at least some of the symptoms of the fibrotic disorder. This method includes the step of contacting the tissue with an agent that modulates expression of one or more differentially expressed genes in the tissue.

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD);

nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1

Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abl-interactor 2 (Abi-2); and other differentially expressed genes disclosed herein.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abl-interactor 2 (Abi-2).

The tissue for use in this method can be any derived from a human or non-human animal. In some embodiments, the tissue is derived from a female reproductive system, *e.g.*, endometrium, or tissue derived from the uterus, cervix, vagina, fallopian tube, or ovary. Because the experiments presented herein relate to human subjects, a preferred tissue sample for the methods of the invention is one derived from a human. Particularly preferred is tissue derived from a subject suspected of having or at risk for developing a fibrotic disorder (such as a woman suspected of having or at risk for developing leoimyoma, endometriosis, ovarian hyperstimulation syndrome, peritoneal adhesions, or other tissue fibroses) based on clinical findings or other diagnostic test results.

The method of the present invention utilizes one or more agents that modulate expression one or more differentially expressed genes in the tissue. Numerous agents for modulating expression of such genes in a tissue are known. Any of those suitable for the particular system being used may be employed. Typical agents for modulating expression of such genes are proteins, nucleic acids, and small organic or inorganic molecules such as hormones (*e.g.*, natural or synthetic steroids). Preferably, the agent is not a hormone.

An example of a protein that can modulate gene expression is an antibody that specifically binds to the gene product. Such an antibody can be used to interfere with the

interaction of the gene product and other molecules that bind the gene product. Products of the differentially expressed genes (or immunogenic fragments or analogs thereof) can be used to raise antibodies useful in the invention. Such gene products (*e.g.*, proteins) can be produced by purification from cells/tissues, recombinant techniques or chemical synthesis as described above. Antibodies for use in the invention include polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library. See, for example, Kohler *et al.*, *Nature*, 1975, 256:495; Kohler *et al.*, *Eur. J. Immunol.*, 1976, 6:511; Kohler *et al.*, *Eur. J. Immunol.*, 1976, 6:292; Hammerling *et al.*, "Monoclonal Antibodies and T Cell Hybridomas," Elsevier, N.Y., 1981; Ausubel *et al.*, supra; U.S. Patent Nos. 4,376,110, 4,704,692, and 4, 946,778; Kosbor *et al.*, *Immunology Today*, 1983, 4:72; Cole *et al.*, *Proc. Natl. Acad. Sci. USA*, 1983, 80:2026; Cole *et al.*, "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1983; and Huse *et al.*, *Science*, 1989, 246:1275.

Other proteins that can modulate gene expression include variants of the gene products that can compete with the native gene products for binding ligands such as naturally occurring receptors of these gene products. Such variants can be generated through various techniques known in the art. For example, protein variants can be made by mutagenesis, such as by introducing discrete point mutation(s), or by truncation. Mutation can give rise to a protein variant having substantially the same, or merely a subset of the functional activity of a native protein. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to another molecule that interacts with the protein. In addition, agonistic (or superagonistic) forms of the protein may be generated that constitutively express one or more functional activities of the protein. Other variants of the gene products that can be generated include those that are resistant to proteolytic cleavage, as for example, due to mutations which alter protease target sequences. Whether a change in the amino acid sequence of a peptide results in a protein variant having one or more functional activities of a native protein can be readily determined by testing the variant for a native protein functional activity (*e.g.*, binding a receptor or inducing a cellular response).

Another agent that can modulate gene expression is a non-peptide mimetic or chemically modified form of the gene product that disrupts binding of the encoded protein to other proteins or molecules with which the native protein interacts. See, *e.g.*, Freidinger *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (*e.g.*, see Huffman *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopepitides (Ewenson *et al. J. Med. Chem.*, 1986, 29:295; and Ewenson *et al.* in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill, 1985), beta-turn dipeptide cores (Nagai *et al. Tetrahedron Lett*, 1985, 26:647; and Sato *et al. J. Chem. Soc. Perkin. Trans.*, 1986, 1:1231), and beta-aminoalcohols (Gordon *et al. Biochem. Biophys. Res. Commun.*, 1985, 126:419; and Dann *et al. Biochem. Biophys. Res. Commun.*, 1986, 134:71). Proteins may also be chemically modified to create derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of proteins encoded by differentially expressed genes can be prepared by linking the chemical moieties to functional groups on amino acid side chains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

The agent that directly reduces expression of the differentially expressed gene can also be a nucleic acid that reduces expression of the gene. For example, the nucleic acid can be an antisense nucleic acid that hybridizes to mRNA encoding the protein. Antisense nucleic acid molecules for use within the invention are those that specifically hybridize (*e.g.* bind) under cellular conditions to cellular mRNA and/or genomic DNA encoding a protein in a manner that inhibits expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

Antisense constructs can be delivered as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes the protein. Alternatively, the antisense construct can take the form of an

oligonucleotide probe generated *ex vivo* which, when introduced into a protein expressing cell, causes inhibition of protein expression by hybridizing with an mRNA and/or genomic sequences coding for the protein. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, *e.g.* exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see, *e.g.*, U.S. Pat. Nos. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol *et al.*, *Biotechniques*, 1988, 6:958-976; and Stein *et al.*, *Cancer Res.*, 1988, 48:2659-2668. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between the -10 and +10 regions of a protein encoding nucleotide sequence, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to mRNA encoding the protein to be inhibited. The antisense oligonucleotides will bind to mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well (Wagner, R., *Nature*, 1994, 372:333). Therefore, oligonucleotides complementary to either the 5 or 3 untranslated, non-coding regions of a differentially expressed gene could be used in an antisense approach to inhibit translation of endogenous mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the

AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of the mRNA, antisense nucleic acids should be at least eighteen nucleotides in length, and are preferably less than about 100 and more preferably less than about 30, 25, 20, or 18 nucleotides in length.

Antisense oligonucleotides of the invention may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouricil, beta-D-galactosylqueosin- e, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-idimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopenten- yladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Antisense oligonucleotides of the invention may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose; and may additionally include at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an alpha-anomeric oligonucleotide. An alpha-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual beta-units, the strands run parallel to each other (Gautier *et al.*, *Nucl. Acids Res.*, 1987, 15:6625-6641). Such oligonucleotide can be a 2'-0-methylribonucleotide (Inoue *et al.*, *Nucl. Acids Res.*, 1987, 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, *FEBS Lett.*, 1987, 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, *etc.*). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al. Nucl. Acids Res.*, 1988, 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85:7448-7451).

The antisense molecules should be delivered into cells that express the differentially expressed (*e.g.*, overexpressed) genes *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA into cells. For instance, antisense molecules can be introduced directly into the tissue site by such standard techniques as electroporation, liposome-mediated transfection, CaCl-mediated transfection, or the use of a gene gun. Alternatively, modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be used.

However, because it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs, a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong promoter (*e.g.*, the CMV promoter). The use of such a construct to transform cells will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous gene transcripts and thereby prevent translation of the mRNA.

Ribozyme molecules designed to catalytically cleave target mRNA transcripts can also be used to prevent translation of mRNA and expression of protein (see, *e.g.*, PCT Publication No. WO 90/11364, published Oct. 4, 1990; Sarver *et al.*, *Science*, 1990, 247:1222-1225 and U.S. Pat. No. 5,093,246). While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy target mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead

ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature*, 1988, 334:585-591. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. Ribozymes within the invention can be delivered to a cell using a vector.

The expression of endogenous genes that are overexpressed in fibrotic disorders can also be reduced by inactivating or "knocking out" the gene or its promoter using targeted homologous recombination. See, *e.g.*, Kempin *et al.*, *Nature*, 1997, 389:802; Smithies *et al.*, *Nature*, 1985, 317:230-234; Thomas and Capecchi, *Cell*, 1987, 51:503-512; and Thompson *et al.*, *Cell*, 1989, 5:313-321. For example, a mutant, non-functional gene variant (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous gene (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the gene *in vivo*.

Alternatively, endogenous gene expression may be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene(s) (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells. (See generally, Helene, C., *Anticancer Drug Des.*, 1991, 6(6):569-84; Helene, C., *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660:27-36; and Maher, L. J., *Bioassays*, 1992, 14(12):807-15).

Antisense nucleic acid, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramide chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Another agent that can be used to modulate gene expression in fibrotic tissue is a hormone. Numerous naturally occurring and synthetic hormones are known to cause physiological changes in such tissue and are available commercially. See, *e.g.*, PDR: Physician's Desk Reference, 2002. Those particular hormones which modulate expression of differentially expressed genes in a given sample tissue can be determined empirically by contacting a series of tissue samples with a panel of different hormones and analyzing the tissue samples for changes in phenotype over time. In experiments relating to the invention, it was shown that GnRHa therapy modulated the expression of 297 genes in leiomyoma and myometrium compared to untreated group ($P<0.02$). In addition, GnRHa, TGF-b and TGF-b receptor type II antisense treatments resulted in differential regulation of 134, 144, and 154 specific genes, respectively ($P<0.005$ and 0.001). The products of these genes were functionally categorized as key regulators of cell cycle, transcription factors, signal transduction, ECM turnover and apoptosis. Based on (i) expression values, (ii) functional classification and (iii) regulation by GnRH and TGF-b mediated actions, we selected 10 of these genes and validated their expression in leiomyoma and myometrium, and in LSMC and MSMC using RealTime PCR, western blotting and immunohistochemistry. In conclusion, the results provide additional evidence for the difference in gene expression profile between leiomyoma and myometrium, and reveal the profile of previously unrecognized novel genes whose expression are the target of GnRH and TGF-β actions in leiomyoma and myometrium.

The agent that can be used to modulate gene expression in fibrotic tissue may be administered to non-human animals or humans in pharmaceutically acceptable carriers (*e.g.*, physiological saline) that are selected on the basis of mode and route of administration and standard pharmaceutical practice. For example, the pharmaceutical compositions of the invention might include suitable buffering agents such as acetic acid or its salt (1-2% w/v); citric acid or its salt (1-3% w/v); boric acid or its salt (0.5-2.5% w/v); succinic acid; or phosphoric acid or its salt (0.8-2% w/v); and suitable preservatives such as benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) or thimerosal (0.004-0.02% w/v). Examples of compositions suitable for parenteral administration include sterile aqueous preparations such as water, Ringer's solution, and isotonic sodium chloride solution. In addition,

sterile, fixed oils might be used as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for local, subcutaneous, intramuscular, intraperitoneal or intravenous administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. The pharmaceutical compositions useful in the invention may be delivered in mixtures of more than one pharmaceutical composition.

The compositions of the invention may be administered to animals or humans by any conventional technique. Such administration might be parenteral (*e.g.*, intravenous, subcutaneous, intramuscular, or intraperitoneal introduction). Preferably, the compositions may also be administered directly to the target site (*e.g.*, a portion of the reproductive tract or peritoneal cavity) by, for example, surgical delivery to an internal or external target site, or by catheter to a site accessible by a blood vessel. Other methods of delivery, *e.g.*, liposomal delivery or diffusion from a device impregnated with the composition, are known in the art. The composition may be administered in a single bolus, multiple injections, or by continuous infusion (*e.g.*, intravenously or by peritoneal dialysis).

The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of response without causing clinically unacceptable adverse effects. Preferred modes of administration include parenteral, injection, infusion, deposition, implantation, anal or vaginal supposition, oral ingestion, inhalation, and topical administration. Injections can be intravenous, intradermal, subcutaneous, intramuscular, or interperitoneal. For example, the pharmaceutical composition can be injected directly into target site for the prevention of fibrotic disorders, such as leiomyoma, endometriosis, ovarian hyperstimulation syndrome, or adhesion formation. In some embodiments, the injections can be given at multiple locations. Implantation includes inserting implantable drug delivery systems, *e.g.*, microspheres, hydrogels, polymeric reservoirs, cholesterol matrixes, polymeric systems, *e.g.*, matrix erosion and/or diffusion systems and non-polymeric systems, *e.g.*, compressed, fused, or partially fused pellets. Inhalation includes administering the pharmaceutical composition with an aerosol in an inhaler, either alone or

attached to a carrier that can be absorbed. For systemic administration, it may be preferred that the pharmaceutical composition is encapsulated in liposomes. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intraperitoneal, intrastemal injection or infusion techniques. In certain preferred embodiments of the invention, the administration can be designed so as to result in sequential exposure of the pharmaceutical composition over some period of time, *e.g.*, hours, days, weeks, months or years. This can be accomplished by repeated administrations of the pharmaceutical composition, by one of the methods described above, or alternatively, by a sustained-release delivery system in which the pharmaceutical composition is delivered to the subject for a prolonged period without repeated administrations. By sustained-release delivery system, it is meant that total release of the pharmaceutical composition does not occur immediately upon administration, but rather is delayed for some period of time. Release can occur in bursts or it can occur gradually and continuously. Administration of such a system can be, *e.g.*, by long-lasting oral dosage forms, bolus injections, transdermal patches, and subcutaneous implants.

A therapeutically effectivc amount is an amount that is capable of producing a medically desirable result in a treated animal or human. As is well known in the medical arts, dosage for any one animal or human depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Toxicity and therapeutic efficacy of the compositions of the invention can be determined by standard pharmaceutical procedures, using cells in culture and/or experimental animals to determine the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Agents that exhibit large therapeutic indices are preferred. While agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of the tissues to be treated in order to minimize potential damage to uninvolved tissue and thereby reduce side effects. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within the range of

circulating concentrations that include an ED50 with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration utilized.

As used herein, the terms "bind," "binds," or "interacts with" mean that one molecule recognizes and adheres to a particular second molecule in a sample, but does not substantially recognize or adhere to other structurally unrelated molecules in the sample. Generally, a first molecule that "specifically binds" a second molecule has a binding affinity greater than about 10^5 to 10^6 moles/liter for that second molecule.

By reference to an "antibody that specifically binds" another molecule is meant an antibody that binds the other molecule, and displays no substantial binding to other naturally occurring proteins other than those sharing the same antigenic determinants as other molecule. The term "antibody" includes polyclonal and monoclonal antibodies as well as antibody fragments or portions of immunolglobulin molecules that can specifically bind the same antigen as the intact antibody molecule.

As used herein, a "nucleic acid," "nucleic acid molecule," "oligonucleotide," or "polynucleotide" means a chain of two or more nucleotides such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid).

The term "subject," as used herein, means a human or non-human animal, including but not limited to mammals, such as a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, rat, and mouse.

The term "differentially expressed gene", as used herein, means a gene that is either over-expressed or underexpressed in fibrotic tissue, compared to normal, non-fibrotic tissue. Accordingly, the method of treatment of the present invention is directed to upregulating the expression of one or more genes that are underexpressed in fibrotic tissue and downregulating the expression of one or more genes that are overexpressed in fibrotic tissue.

When referring to a differentially expressed gene, the phrase "modulates the expression of" means upregulates or downregulates the amount or functional activity of the gene, or otherwise modifies the availability of the gene product to interact with a receptor.

The terms, "treat", "treatment", and "treating", as used herein, are intended to include the prevention of a fibrotic disorder and partial or full alleviation of an existing fibrotic disorder within a subject.

Materials and Methods

Tissues. Portions of leiomyoma and matched myometrium were collected from premenopausal women (N=6) who were scheduled to undergo hysterectomy for indications related to symptomatic leiomyomas. Three of the patients received GnRHa therapy for three months prior to surgery. The untreated patients did not receive any medications (including hormonal therapy) during the previous 3 months prior to surgery, and based on endometrial histology and the patient's last menstrual period they were from early-mid secretory phase of the menstrual cycle. To maintain a standard, all leiomyomas selected for this study were between 2 to 3cm in diameter. Following collection, the tissues were divided into several pieces and either immediately snap frozen and stored in liquid nitrogen for further processing, fixed and paraffin embedded for histological evaluation and immunohistochemistry, or used for isolation of leiomyoma and myometrial smooth muscle cells and culturing (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61). The tissues were collected at the University of Florida affiliated Shands Hospital with prior approval obtained from the Institutional Review Board.

Isolation and Culture of Leiomyoma and Myometrial Smooth Muscle Cells. To determine the direct action of GnRHa on global gene expression in leiomyoma and myometrial smooth muscle cells (LSMC and MSMC), the cells were isolated and cultured as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8). Only untreated tissues were used for isolation of LSMC and MSMC. Prior to use in these experiments, the primary cell cultures were seeded in 8-well culture slides (Nalge Nunc, Naperville, IL) and after 24 hrs of culturing they were characterized using immunofluroscence microscopy and antibodies to α smooth muscle actin, desmin and vimentin (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61). LSMC and MSMC were cultured in 6-well plates at an approximate

density of 10^6 cells/well in DMEM-supplemented media containing 10% FBS. After reaching visual confluence, the cells were washed in serum-free media and incubated for 24hrs under serum-free, phenol red-free condition (Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8). The cells were then treated with 0.1 μM of GnRHa (leuprolide acetate, Sigma Chemical, St Louis, MO) for a period of 2, 6 and 12 hours (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)).

cDNA Microarray and Gene Expression Profiling. Total cellular RNA was isolated from the tissues and cells using Trizol (Invetrogen, Carlsbad, CA). The isolated RNA was treated with DNase I (Roche, Molecular Biochemicals, Indianaplis, IN) at 1 unit/10 µg of RNA for 20 min at 25°C, heat-inactivated at 75°C and subjected to further purification using RNeasy Kit (Qiagen, Valencia, CA). The RNA was then subject to amplification by reverse transcription using SuperScript Choice system (Invitrogen), with final concentrations in 20 µl first-strand reaction of 100 pmol of high performance liquid chromatography-purified T7-(dT)24 primer (Genset Corp, La Jolla, CA.), 8 µg of RNA, 1× first-strand buffer, 10 mM dithiothreitol, 500µM of each dNTP, and 400 units of Superscript II reverse transcriptase (T7 Megascript kit; Ambion, Austin, TX). The second-strand cDNA synthesis was performed in a 150 µl reaction consisting of, at the final concentrations, 1× second-strand reaction buffer, 200 µM each dNTP, 10 units of DNA ligase, 40 units of DNA polymerase I, and 2 units of RNase H (Invitrogen), and double-stranded cDNA was purified by phenol:chloroform extraction using phase lock gels (Eppendorf-5 Prime, Inc. Westbury, NY) and an ethanol precipitation (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71).

Five micrograms of purified cDNA was reverse transcribed using Enzo BioArray high yield RNA transcript labeling kit (Affymetrix, Santa Clara, CA) and the product was purified in RNeasy spin columns (Qiagen) according to manufacture's instructions. Following an overnight ethanol precipitation, cRNA was re-suspended in 15 µl of diethyl pyrocarbonate-treated water (Ambion) and quantified using a Beckman DU530 Life Science UV-visible spectrophotometer. Following quantification of cRNA to reflect any carryover of unlabeled total RNA according to an equation given by Affymetrix (adjusted cRNA yield = cRNA (μg) measured after in vitro transcription (starting total RNA) (fraction of cDNA reaction used in in vitro transcription), 20µg

of cRNA was fragmented (0.5μg/μl) according to Affymetrix instructions using the 5× fragmentation buffer containing 200 mM Tris acetate, pH 8.1, 500 mM potassium acetate and 150 mM magnesium acetate (Sigma Chemical, St. Louis, MO). 20 μg of the adjusted fragmented cRNA was added to a 300 μl of hybridization mixture containing at final concentrations 0.1 mg/ml herring sperm DNA (Promega/Fisher, Madison, WI), 0.5 mg/ml acetylated bovine serum albumin (Invitrogen), and 2× MES hybridization buffer (Sigma). 200 μl of the mixture was hybridized to the human U95A Affymetrix GeneChip arrays, purchased at the same time from the same lot number and used within two weeks of purchase in order to maintain standard. In addition, an aliquot of random samples were first hybridized to an Affymetrix Test 2 Array to determine sample quality according to manufacturer's criteria. After meeting recommended criteria for use of the expression arrays, the hybridization was performed for 16 hrs at 45°C, followed by washing, staining, signal amplification with biotinylated anti-strepavidin antibody, and the final staining step according to manufactures protocol.

Microarray Data Analysis. The Chips were scanned to obtain the raw hybridization values using Affymetrix Genepix 5000A scanner. Difference in the levels of fluorescence spot intensities representing the rate of hybridization between the 25 basepair oligonucleotides and their mismatches were analyzed by multiple decision matrices to determine the presence or absence of gene expression, and to derive an average difference score representing the relative level of gene expression. The fluorescence spot intensities, qualities and local background were assessed automatically by Genepix software with a manual supervision to detect any inaccuracies in automated spot detection. Background and noise corrections were made to account for nonspecific hybridization and minor variations in hybridization conditions. The net hybridization values for each array were normalized using a global normalization method as previously described (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71). To identify the changes in pattern of gene expression, the average and standard deviation (SD) of the globally normalized values were calculated followed by subtraction of the mean value from each observation and division by the SD. The mean transformed expression value of each gene in the transformed data set was set at 0 and the SD at 1 (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71).

The transformed gene expression values were subjected to Affymetrix Analysis Suite V 5.0. Briefly, probe sets that were flagged as absent on all arrays using default settings were removed from the datasets. After application of this filtering, the dataset was reduced from 12,625 probe sets to 8580 probe sets. The gene expression value of the remaining probe sets was then subjected to unsupervised and supervised learning, discrimination analysis, and cross validation (Eisen, MB *et al. Proc Natl Acad Sci USA*, 1998, 95:14863-14868; Varela, JC *et al. Invest Ophthalmol Vis Sci*, 2002, 43:1772-1782; Tusher, VG *et al. Proc Natl Acad Sci USA*, 2001, 98:5116-5121; Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). After variation filtering, the coefficient of variation was calculated for each probe set across all chips and the probe sets were ranked by the coefficient of variation of the observed single intensities. The expression values of the selected genes were then subjected to R programming analysis that assesses multiple test correction to identify statistically significant gene expression values (Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). The gene expression values having a statistical significance of $p \leq 0.02$ (ANOVA, Tukey test) between leiomyoma and myometrium from GnRH-treated and untreated cohorts, and $p \leq 0.005$ between GnRHa-treated and untreated cells (control) were selected. The validity of gene sets identified at these p values in predicting treatment class was established using "leave-one-out" cross validation where the data from one array was left out of the training set and probe sets with differential hybridization signal intensities were identified from the remaining arrays (Varela, JC *et al. Invest Ophthalmol Vis Sci*, 2002, 43:1772-1782; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). Hierarchical clustering and K-means analysis was performed and viewed with the algorithms in the software packages Cluster and TreeView (Eisen, MB *et al. Proc Natl Acad Sci USA*, 1998, 95:14863-14868).

Gene Classification and Ontology Assessment. The selected differentially expressed and regulated genes in the above cohorts were subjected to functional annotation and visualization using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis G Jr. *et al.*, DAVID: Database for Annotation, Visualization, and Integrated Discovery,

Genome Biology, 2003; 4(5):P3; Hosack D.A. *et al.*, Glynn Dennis Jr, Brad T Sherman, HClifford Lane, Richard A Lempicki. Identifying Biological Themes within Lists of Genes with EASE, Genome Biology, 2003, 4(6):P4). The integrated GoCharts assigns genes to specific ontology functional categories based on selected classifications, KeggCharts assigns genes to KEGG metabolic processes and context of biochemical pathway maps, and DomainCharts assigning genes according to PFAM conserved protein domains.

Quantitative RealTime PCR. Realtime PCR was utilized for verification of 10 differentially expressed and regulated genes identified in leiomyoma and myometrium as well as LSMC and MSMC from untreated and GnRHa-treated cohorts. The selection of these genes was based not only on their expression values (up or downregulation), but classification and biological functions important to leiomyoma growth and regression, regulation by ovarian steroids, GnRHa and TGF-β (Luo, X *et al.* 2004 Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to TGF-b autocrine/paracrine action. Accompanying paper) as indicated in the literature. They are IL-11, CITED2, Nur77, EGR3, TGIF, TIEG, p27, p57, GAS-1 and GPRK5 representing cytokines, transcription factors, cell cycle regulators and signal transduction. Realtime PCR was carried out as previously described using Taqman and ABI-Prism 7700 Sequence System and Sequence Detection System 1.6 software (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)). Results were analyzed using the comparative method and following normalization of expression values to the 18S rRNA expression according to the manufacturer's guidelines (Applied Biosystems) as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)).

Western Blot Analysis and Immunohistochemical Localization. For immunoblotting, total protein was isolated from small portions of GnRHa-treated and untreated leiomyoma and myometrium as well as the GnRHa-treated and untreated cells as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16). Following determination of the tissue homogenates and cell lysates protein content an equal amount of sample proteins were subjected to SDS-PAGE and transferred to polyvinyldiene difluoride (PVDF) membrane. The blots were incubated with anti-TIEG antibody, kindly provided by Dr. Thomas Spelsberg, Department of Biochemistry, Mayo Clinic,

Rochester, MN (Johnsen, SA *et al. Oncogene*, 2002, 21:5783-90), TGIF, EGR3, p27, p57, Nur77 and Gas1 antibodies purchased from Santa Cruz Biochemical (Santa Cruz, CA), IL-11 antibodies purchased from R & D system (Minneapolis, MN) for 1 hr at room temperature. The membranes were washed, exposed to corresponding HRP-conjugated IgG for 1 hr and immunostained proteins were visualized using enhanced chemiluminesence reagents (Amersham-Pharmacia Biotech, Piscataway, NJ) as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61).

For immunohistochemical localization, tissue sections were prepared from formalin-fixed and paraffin embedded leiomyoma and myometrium. Tissue sections were microwave prior to immunostaining using antibodies to IL-11, TGIF, TIEG, EGR3, Nur77, p27, p57 and Gas1. The antibodies were used at concentrations of 5 μg of IgG/ml for 2-3 hrs at room temperature. Following further processing including incubation with biotinylated secondary antibodies and avidin-conjugated HRP (ABC Elite kit, Vector Laboratories, Burlingame, CA), the chromogenic reaction was detected with 3,3'-diaminobenzidine tetrahydrochloride solution. In some instances the slides were counter stained with hematoxylin. Omission of primary antibodies or incubation of tissue sections with non-immune mouse IgG instead of primary antibodies at the same concentration during immunostaining served as controls (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61).

<u>Determination of TGF-β1 on global gene expression in LSMC and MSMC</u>. All the materials utilized for this study including isolation of leiomyoma and myometrial cells are identical to those described in detail above. To determine the effect of TGF-β1 on global gene expression in LSMC and MSMC, the cells were cultured in 6-well plates at approximate density of 10^6 cells/well in DMEM-supplemented media containing 10% FBS. After reaching visual confluence the cells were washed in serum-free media and incubated for 24 hrs under serum-free, phenol red-free condition (10,11). The cells were then treated with 2.5 ng/ml of TGF-β1 (R & D System, Minneapolis, MI) for 2, 6 and 12 hrs. To further profile the autocrine/paracrine action of TGF-β1 on gene expression in LSMC and MSMC, the cells were cultured as above and

treated with 1 μM of TGF-β type II receptor antisense or sense oligonucleotides for 24 hrs as previously described (10,11). The cells were washed and then treated with TGF-β_1 (2.5 ng/ml) for 2 hrs. Parallel experiments using untreated cells were used as controls including an additional control for TGF-β type II receptor antisense and sense experiments.

Total cellular RNA was isolated from LSMC- and MSMC-treated and untreated controls and subjected to microarray analysis with detailed description of all procedures provided in the accompanying manuscript (22). To maintain standard and allow for comparative analysis, the GeneChips used in this study were utilized, simultaneously processed and their gene expression values were subjected to global normalization and transformation with the GeneChips used in the accompanying manuscript (22). Following these unsupervised assessments the coefficient of variation was calculated for each probe set across all the chips used in this and other study (22), and the selected gene expression values of this study were independently subjected to supervised learning including R programming analysis and ANOVA with false discovery rate selected at $p \leq 0.001$ (22,24,25). The genes identified in these cohorts were analyzed for functional annotation and visualized using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software with integrated GoCharts as described in detail (22). Following the analysis, we selected 12 of the differentially expressed and regulated genes, including 10 identified and validated in leiomyoma and myometrium from untreated and GnRHa-treated cohorts, as well as LSMC and MSMC treated *in vitro* with GnRHa (22), for validation in response to TGF-β-time dependent action using Realtime PCR. They include IL-11, EGR3, CITED2, TIEG, TGIF, Nur77, p27, p57, GAS-1 and GPRK5. In addition, the expression of Runx1 and Runx2, transcription factors that interact with TGF-β receptor signaling pathways (26), was validated in LSMC and MSMC as well as in leiomyoma and myometrium from GnRHa-treated and untreated cohorts. Detail description of the materials and methods for Realtime PCR as well as data analysis is provided in the accompanying manuscript (21,22).

Example 1—Gene Expresssion Profiles in Leiomyoma and Normal Myometrium

Global gene expression profiling has been instrumental in identifying the molecular environment of tissues with respect to fingerprints of their physiological and pathological

behavior, and *in vitro* cellular responses to various regulatory molecules. The present inventors used this approach and characterized the gene expression profile of leiomyoma and matched myometrium, and their transcriptional changes in response to hormonal transition induced by GnRHa therapy. The initial assessment of the gene expression values in leiomyoma, myometrium and their isolated smooth muscle cells from untreated as well as GnRHa- and TGF-β-treated (Luo, X *et al.* 2004 Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to TGF-b autocrine/paracrine action. Accompanying paper) cohorts revealed a uniform expression of transcripts for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase, α-actin and a large number of ribosomal proteins, indicating that the expression profile is consistent with established standards for gene expression analysis. Following global normalization and transformation of the gene expression values, supervised learning, discrimination analysis, cross validation and variation filtering, the gene expression values were subjected to R programming analysis and ANOVA with false discovery rate selected at $p \leq 0.02$.

Using the above analysis, the present inventors identified a total of 153 genes, including 19 EST, or 1.23% of the genes, and 122 genes including 21 EST or 0.98% of the genes on the array, as differentially expressed in leiomyoma compared to matched myometrium from untreated and GnRHa-treated tissues, respectively. Hierarchical clustering and Tree-View analysis separated the genes in each cohort into distinctive clusters with sufficient variability allowing division into their respective subgroups (Figure 1). Of the 153 (excluding 19 EST) differentially expressed genes in untreated cohorts, 82 were upregulated and 52 downregulated in leiomyoma compared to myometrium (Table 1). Of the 122 genes (excluding 21 EST) in leiomyoma and myometrium from patients who received GnRHa therapy, 34 transcripts were upregulated and 67 downregulated, in leiomyoma compared to myometrium, respectively (Table 2). Analysis of the variance-normalized mean (K-means) separated the differentially expressed and regulated genes in these cohorts into 4 distinctive clusters, with genes in clusters A and D displaying a tissue-specific response, while genes in cluster B and C showing regulatory response to GnRHa therapy (Fig. 2). To further characterize the molecular environment of leiomyoma from myometrium and their response to GnRHa therapy, we compared the gene expression profiles in GnRHa-treated with corresponding untreated tissues. The analysis indicated that the expression of 170

(excluding 26 EST) and 167 (excluding 31 EST) genes are targeted by GnRHa therapy in leiomyoma and myometrium, compared to their respective untreated cohorts (Tables 3 and 4). Of these genes, 96 and 89 transcripts were downregulated in leiomyoma and myometrium, respectively, due to GnRHa therapy, compared to their respective untreated tissues, with 3 transcripts were commonly found among the tissues in these cohorts, with different regulatory pattern of expression (compare Tables 3 and 4).

Table 1. Categorical list of differentially expressed genes identified in leiomyoma compared to matched myometrium. The genes were identified following unsupervised and supervised analysis of their expression values and subjected to R programming environment and ANOVA with a false-discovery rate of rate of $p \leq 0.02$ as described in materials and methods. Of the 153 genes identified as differentially expressed, 82 genes were up (+) and 52 genes were downregulated (-) in leiomyoma compared to myometrium excluding 19 EST.

Table 2. Categorical list of differentially expressed genes identified in leiomyoma compared to myometrium in response to GnRHa therapy. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.02$. Of the 122 genes identified, the expression of 34 genes was up (+) and 67 gene downregulated (-) in GnRH-treated leiomyoma (LYM) compared to myometrium (MYM) excluding 21 EST).

Table 3. Categorical list of differentially expressed genes identified in leiomyoma from GnRHa-treated compared to untreated leiomyoma. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.02$. Of the 170 genes identified, the expression of 74 genes was up (+) and 96 genes downregulated (-) in GnRH-treated compared to untreated leiomyoma (LMY) excluding 26 EST.

Table 4: Categorical list of differentially expressed genes identified in myometrium from GnRHa-treated compared to untreated myometrium. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.02$. Of the 167 genes identified, the expression of 47 genes was up (+) and 89 genes downregulated (-) in GnRH-treated compared to untreated myometrium (MYM) excluding 31 EST.

A few microarray studies have reported the gene expression profile of leiomyoma and myometrium (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). The present inventors performed a comparative analysis using the differentially expressed genes identified in the untreated leiomyoma and matched myometrium of this study, with the list of genes reported in four of the other studies, searching for a set of commonly expressed genes. The comparison identified 2 genes in this study in common with at least one of the other studies. However, lowering the false discover rate to $p \leq 0.05$ enabled the identification of a larger number of genes (422 including 49 EST), of which 11 transcripts were found in common with other studies (Table 5).

Table 5: The list of common genes found in our study of leiomyoma and matched myometrium from early-med secretory phase of the menstrual cycle following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.05$ to allow comparison with the results of four other microarray studies utilizing leiomyoma and myometrium from proliferative and secretory phases of the menstrual cycle.

Gene ontology assessment and division of differentially expressed genes into similar functional categories indicated that the products of a large percentage of these genes (40% to 67%), in leiomyoma and myometrium from both GnRHa treated and untreated cohorts, are involved in metabolic processes, catalytic activities, binding, signal transduction, transcriptional and translational activities, cell cycle regulation, cell and tissue structure, *etc.* (Fig. 3, Tables 1-4). In

addition, 15% to 23% of the genes were either functionally unclassified, or their roles in biological process are still unknown.

Example 2—Time-Dependent action of GnRHa on Gene Expression Profile of Leiomyoma and Myometrial Smooth Muscle Cells (LSMC and MSMC)

Leiomyoma and myometrium and their smooth muscle cells (LSMC and MSMC) express GnRH and GnRH receptors, and GnRH through the activation of specific signal transduction pathways results in transcriptional regulation of several genes downstream from these signals in LSMC and MSMC (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61. To obtain a comprehensive picture of transcriptional changes induced by GnRHa direct action in leiomyoma and myometrium, we isolated LSMC and MSMC from the untreated cohorts. The serum starved LSMC and MSMC were treated with GnRHa (0.1 μM) for 2, 6 and 12 hours and their isolated RNA was subjected to microarray analysis. Based on the same data analysis criteria described above with a false discovery rate of $p \leq 0.005$, we identified 281 genes including 36 EST or 2.2% of the genes on the array displaying differential expression and regulation in LSMC and MSMC in response to GnRHa treatment in a time-dependent manner compared to untreated controls (Fig. 4). Hierarchical clustering analysis also separated these genes into different clusters in response to time-dependent action of GnRHa in LSMC and MSMC, with expression patterns sufficiently different to cluster into their respective subgroups (Figs. 4). Analysis of the variance-normalized mean (K-means) separated the differentially expressed and regulated genes in these cohorts into 4 distinctive clusters, with genes in clusters A and D displaying a cell-specific response, while genes in cluster B and C showing regulatory behaviors to GnRHa time-dependent action (Fig. 5). Among the differentially expressed and regulated genes, the transcripts of 48 genes were identified as commonly expressed in LSMC and the original tissues (leiomyoma) from the untreated cohort used (Table 6).

Table 6: Categorical list of differentially expressed genes in leiomyoma from GnRHa treated and LSMC treated with GnRHa for 2, 6 and 12 hours. The genes were identified

following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.005$. Of the 130 genes identified, the expression of 34 genes was up- (+) and 96 genes downregulated (-) excluding 26 EST.

Gene ontology and functional annotation of the differentially expressed and regulated genes into similar functional categories indicated that in LSMC and MSMC, similar to their original tissues, the majority of the gene products are involved in cellular processes, catalytic activities, binding, signal transduction, transcriptional and translational activities, metabolism, cell cycle regulation and cellular structure (Figs. 6). The time-dependent action of GnRHa on the expression of a selective group of genes representing growth factors/cytokines/chemokines/receptors, intracellular signal transduction pathways, transcription factors, cell cycle, cell adhesion/receptor/ECM/cytoskeleton in LSMC and MSMC are shown in Figure 7.

<u>Example 3—Verification of Gene Transcripts in Leiomyoma, Myometrium and LSMC and MSMC</u>

Among the differentially expressed and regulated genes identified in these tissues and cells, we selected 10 genes for verification using Realtime PCR, western blotting and immunohistochemistry. The selection of these genes was based not only on their expression values (up or downregulated), but also on gene classification, biological functions important to leiomyoma growth and regression, and regulation by ovarian steroids, GnRH and TGF-β (Luo, X *et al.* 2004 Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to TGF-b autocrine/paracrine action. Accompanying paper)) as indicated in the literature. The genes selected for validation were IL-11, CITED2, Nur77, EGR3, TGIF, TIEG, CDKN1B (p27), CDKN1C (p57), GAS-1 and GPRK5, representing cytokines, transcription factors, cell cycle regulators, and signal transduction. The pattern of expression of these genes in leiomyoma and myometrium from untreated and GnRHa-treated cohorts (Fig. 8), as well as in LSMC and MSMC treated with GnRHa for 2, 6 and 12 hrs (Fig. 9) as determined by Realtime PCR, closely overlapped with their expression profiles identified by the microarray analysis.

Western blotting also indicated that leiomyoma and myometrium, as well as LSMC and MSMC locally produce IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 proteins are produced by (Fig. 10). Immunohistochemically, IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 were localized in various cell types in leiomyoma and myometrium, including LSMC and MSMC (Fig. 11). The present inventors did not have access to antibody to GPRK5 and have not yet attempted to quantitate the level of IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 production in leiomyoma and myometrium as well as in LSMC and MSMC in response to GnRHa treatment. However, these results provided further support for the microarray and Realtime PCR data, indicating that various cells types contribute to overall expression of these genes in leiomyoma and myometrium. In addition to these genes, the expression of 15 more genes was validated with Realtime PCR including CTGF, Abl-interactor 2 (Abi2), fibromodulin, Runx1 and Runx2 (Luo, X *et al.* 2004 Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to TGF-b autocrine/paracrine action. Accompanying paper; Levens, E *et al.* "Differential Expression of fibromodulin and Abl-interactor 2 in leiomyoma and myometrium and regulation by gonadotropin releasing hormone analogue (GnRHa) therapy" *Fertil Steril*, 2004, (In press)).

Uterine leiomyoma affect 30 to 35% of women during their reproductive years and up to 70 to 80% before menopause (Baird, DD *et al. Am. J Obstet Gynecol*, 2003, 188: 100-107). The etiology of leiomyoma remains unknown, however they are thought to derive from the transformation of MSMC and/or connective tissue fibroblasts, and display high sensitivity to ovarian steroids for their growth. For this reason, GnRHa therapy is often used for medical management of symptomatic leiomyomas. In addition to GnRHa therapy, clinical and preclinical assessments of selective estrogen and progesterone receptor modulators, either alone, or in combination with GnRHa therapy, have shown efficacy in leiomyoma regression (Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32). Despite their prevalence and the efficacy of these therapies for their medical management, the molecular environment differentiating leiomyoma from adjacent myometrium, and their response to the above therapies is unknown. In the present study, the present inventors characterized gene expression fingerprints of leiomyoma and matched

myometrium from the early-mid secretory phase of the menstrual cycle, a period associated with their rapid growth, their response to hormonal transition induced by GnRHa therapy, and to direct action of GnRHa in isolated LSMC and MSMC prepared from the untreated tissues.

Combining global normalization and unsupervised assessment of the gene expression values derived from all the cohorts enabled us to sort potential candidate genes prior to their putative identification in each cohort. Transcripts of many of the genes on the array were found in leiomyoma and myometrium as well as in LSMC and MSMC. However, leiomyoma/LSMC were not distinguished as a single class from myometrium/MSMC based on single gene markers uniformly expressed only in leiomyoma and/or myometrium. This is not unique to leiomyoma/myometrium since many large-scale gene expression profiling studies have shown the existence of a significant degree of shared gene expression between various tumors and their normal tissue counterparts. However, supervised assessment and multiple test correction in R programming environment (Tusher, VG *et al. Proc Natl Acad Sci USA*, 2001, 98:5116-5121; Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960) with reduced false discovery rate, allowed the identification of a specific set of differentially expressed and regulated genes in descending order of significance in each cohort. The analysis separated these genes into several clusters with a sufficient difference allowing their subdivision into their respective subgroup in leiomyoma, myometrium, their isolated cells, as well as due to GnRHa therapy at the tissue and cellular levels. We identified 153 genes (excluding 19 EST) in these cultures as differentially expressed in leiomyoma compared to myometrium, of which 82 genes were upregulated and 52 downregulated in leiomyoma. GnRHa therapy affected the expression of 122 genes (excluding 21 EST), with 34 upregulated and 67 downregulated genes in leiomyoma compared to myometrium. However, their gene profiles in untreated and GnRHa treated leiomyoma/myometrium differed substantially, pointing out a unique molecular environment that is targeted by GnRHa therapy. Analysis of the variance-normalized mean gene expression values divided these genes into 4 clusters with two clusters showing treatment-specific, while other clusters displayed a tissue-specific response to GnRHa therapy. A similar behavior was also observed with gene clusters identified in LSMC and MSMC in response to GnRHa action in

vitro. The significance of these findings are related to clinical observations indicating that GnRHa therapy affects both leiomyoma and myometrium, with non-myoma tissue regressing more in response to therapy (Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223). The gene expression profiling disclosed herein supports the clinical observations, and further points out that GnRHa therapy targets different genes in leiomyoma and myometrium although they may group in a similar functional category. The recent microarray study using a small-scale array containing probe sets of 1200 known genes (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71) provides support for the current study; however, the present inventors are not aware of any other study using a large-scale gene expression profiling in leiomyoma and myometrium from women who received GnRHa therapy for further comparison.

Since this study was completed, a few other microarray studies have reported the gene expression profiles of leiomyoma and myometrium from the proliferative and secretory phases of the menstrual cycle (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). To broaden the scope of this study, the present inventors compared the genes list identified in untreated leiomyoma and matched myometrium of the present study, with the data sets reported in four of these otherstudies (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). This comparison resulted in identification of only a few genes in common among these studies. Although intrinsic individual tissue variation may contribute toward differences among these studies, standard of experimental process, utilization of different microarry platforms, utilization of tissues from different phases of the menstrual cycle, differences of leiomyoma size, and most importantly the method of data acquisition and analysis (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108) are among other key contributing factors accounting for different study results (Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002 1:951-960). To maintain a standard, the present inventors used

leiomyoma of uniform sizes (2-3 cm in diameters) and matched myometrium, and the untreated cohorts were collected from the early-mid secretory phase of the menstrual cycle, a period associated with leiomyoma maximum growth. However, lowering the false discovery rate of the present study allowed the identification of more transcripts and the appearance of additional common genes with other studies (see Table 5; Refs. Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). Considering the presence of a large number of probe sets on these arrays (i.e. 6800-12,500), selection of genes based only on fold change (Tsibris, JCM *et al. Fertil Steril*, 2002), or higher statistical levels (Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108) is no better than what one would expect by chance alone (Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002 1:951-960). Since the present inventors employed a similar data analysis, a larger number of genes was found in common with our previous microarray study which used only a small-scale array containing about 1200 known genes (Chegini, N *et al. et al. J Soc Gynecol Investig*, 2003, 10:161-71). The present inventors recognize that exclusion of moderately regulated genes during microarray data analysis does not reflect lack of functional importance, since a number of genes previously identified in leiomyoma and myometrium by conventional methods are not included among the differentially expressed genes in our study and other reports (Chegini, N Implication of growth factor and cytokine networks in leiomyomas. In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). However, the expression of newly identified genes requires verification, and their regulation would allow linking their potential biological functions in leiomyoma growth and regression.

GnRHa therapy and most recently SERM and SPRM have been utilized for medical management of leiomyoma (Takeuchi, H *et al. J Obstet Gynaecol Res*, 2000, 26:325-331; Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32; Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223). Unlike SERM and SPRM that act directly on estrogen/progesterone sensitive tissues such as the uterus (Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32), GnRHa is traditionally believed to act primarily at the level of the pituitary-gonadal axis to implement its therapeutic benefits (Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128). However, identification of GnRH and GnRH receptors in several peripheral tissues, including leiomyoma, has led the present inventors to propose an autocrine/paracrine role for GnRH, and an additional site of action for GnRHa therapy (Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61). *In vitro* studies have provided evidence for direct action of GnRHa on several cell types derived from these tissues resulting in alterations of a wide range of cellular activities, including cell growth, apoptosis and gene expression (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255). Using isolated LSMC and MSMC prepared from the untreated tissues allowed the present inventors to identify novel regulatory functions for GnRHa in leiomyoma and myometrium, and discover a wide range of genes whose expression has not previously been recognized to be the target of GnRHa direct action. Similar to their distinct clustering at tissue levels, the differentially expressed and regulated genes identified in LSMC and MSMC were also divided into clusters according to time-dependent response to GnRHa action. The genes in these clusters were either rapidly induced by GnRHa treatment, or required prolong exposure, while others displayed biphasic patterns of temporal regulation in both treatment- and cell- specific fashions. Despite differences in their profiles, substantial similarity existed in functional grouping of

the genes affected by GnRHa therapy in leiomyoma/myometrium, and GnRHa direct action on LSMC/MSMC (*in vitro*), with the expression of 48 genes commonly identified in tissues and cells. The present inventors propose that the hypoestrogenic condition created by GnRHa therapy and contributions of other cell types to overall gene expression at the tissue level may account for the difference in profiles of gene expression between tissues and cell cultures. Gene ontology and division into similar functional categories indicated that the products of the majority of the genes in these clusters are involved in transcriptional and signal transduction activities, cell cycle regulation, extracellular matrix turnover, cell-cell communication, transport and enzyme regulatory activities.

Among the genes in these functional categories are several growth factors, cytokines and chemokines, and polypeptide hormones, identified as differentially expressed in leiomyoma, myometrium and their isolated smooth muscle cells, and were the target of GnRHa action *in vivo* and *in vitro*. Using several conventional methods, previous reports have documented the expression of PDGF, EGF, IGFs, VEGF, FGF, TGF-βs, CTGF, TNF-α, IFN-γ, MCP-1 and IL-8 as well as some of their receptors in leiomyoma and myometrium (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Wu, X *et al. Acta Obstet Gynecol Scand*, 2001, 80:497-504; Senturk, LM *et al. Am J Obstet Gynecol*, 2001, 184:559-566; Sozen, I *et al. Fertil Steril*, 1998, 69:1095-1102). However, the expression of some of these and other genes in this category did not meet the selection criteria of this study, a common discrepancy often observed in microarray analysis, particularly in identifying moderately expressed and regulated genes (Varela, JC *et al. Invest Ophthalmol Vis Sci*, 2002, 43:1772-1782; Tusher, VG *et al. Proc Natl Acad Sci USA*, 2001, 98:5116-5121; Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). For example, the expression of TGF-β isoforms, TGF-β receptors and components of their signaling pathway that are well documented in leiomyoma and myometrium, as well as in their isolated smooth muscles cells (Chegini, N *et*

al. Mol Cell Endocrinol, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Arici, A and Sozen, I *Fertil Steril*, 2000, 73:1006-1011; Lee, BS and Nowak, RA *J Clin Endocrinol Metab*, 2001, 86:913-920), are not consistently identified in microarray studies (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108), although in the current, accompanied (Luo, X *et al.* 2004 Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to TGF-b autocrine/paracrine action. Accompanying paper) and previous (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71) studies we identified most of the members of TGF-β system. Among the cytokines whose expression was identified and validated in the present study is IL-11. IL-11 is recognized to play key regulatory functions in inflammation, angiogenesis and tissue remodeling (Leng, SX and Elias, JA *Int J Biochem Cell Biol*, 1997, 29:1059-62; Tang, W *et al. J Clin Invest*, 1996, 98:2845-53; Zhu, Z *et al. Am J Respir Crit Care Med*, 2001, 164:S67-70; Zimmerman, MA *et al. Am J Physiol Heart Circ Physiol*, 2002, 283:H175-80; Bamba, S *et al. Am J Physiol Gastrointest Liver Physiol*, 2003, 285:G529-38), events that are central to leiomyoma pathophysiology. IL-11 is a member of the IL-6 family and produced by various cell types, including the uterus, and its overexpression is reported to cause sub-epithelial airway fibrosis particularly through interaction with IL-13 and TGF-β (Leng, SX and Elias, JA *Int J Biochem Cell Biol*, 1997, 29:1059-62; Tang, W *et al. J Clin Invest*, 1996, 98:2845-53; Zhu, Z *et al. Am J Respir Crit Care Med*, 2001, 164:S67-70; Zimmerman, MA *et al. Am J Physiol Heart Circ Physiol*, 2002, 283:H175-80; Bamba, S *et al. Am J Physiol Gastrointest Liver Physiol*, 2003, 285:G529-38; Karpovich, N *et al. Mol Hum Reprod*, 2003, 9:75-80). Evidence has been provided that IL-11, similar to TGF-β and IL-13, is overexpressed in leiomyoma compared to myometrium and GnRHa therapy suppressed their expression in these tissues (Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Ding, L *et al. J Soc Gyncol Invest*, 2004,

00, 00). At the cellular level, unlike the expression of TGF-β and IL-13, GnRHa increased IL-11 expression in LSMC and MSMC within 2 to 6 hrs of treatment, which sharply declined to control levels after 12 hrs. Although the nature of differential regulation of IL-11 at the tissue and cellular levels requires detailed investigation, prolonged treatment with GnRHa, the contribution of other cell types and the influence of other autocrine/paracrine regulators, may account for the difference in IL-11 expression between *in vivo* and *in vitro* conditions.

Other differentially expressed and regulated genes identified in the present study functionally belong to signal transduction pathways that are recruited and activated by various growth factors/cytokines/ chemokines, polypeptide hormones, extracellular matrix and adhesion molecules. However the expression of few of these components and other signal transduction pathways has been documented in leiomyoma and myometrium (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Orii, A *et al. J Clin Endocrinol Metab*, 2002, 87:3754-9), and little is known about their recruitment and activation in LSMC and MSMC. The expression of Smads, MAPK and FAK has been identified in leiomyomas and myometrium and evidence has been provided for their regulation and activation by GnRHa in LSMC and MSMC (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6). Here, the present inventors validated the expression of GPRK5 identified as one of the differentially expressed and regulated genes in leiomyoma and myometrium and demonstrated that GnRHa therapy, and *in vitro* treatment of LSMC and MSMC with GnRHa inhibits GPRK5 expression. G-protein-coupled receptor kinases (GPRKs), consisting of six members GPRK1 to GPRK6, act as key regulators of signaling via GPRKs, and are widely expressed in various tissues and cells (Mak, JC *et al. Eur J Pharmacol*, 2002, 436:165-72; Simon, V *et al. Endocrinology*, 2001, 142:1899-905; Simon, V *et al. Endocrinology*, 2003, 144:3058-66; Krasel, C *et al. J Biol Chem*, 2001, 276:1911-1915). Previous studies have demonstrated that pregnant and non-pregnant human myometrium as well as cultured myometrial cells express GPRK2, GPRK4γ and GPRK5, however GPRK3 and

GPRK4α, β, and δ were not detected in myometrium (Simon, V *et al. Endocrinology*, 2001, 142:1899-905; Simon, V *et al. Endocrinology*, 2003, 144:3058-66). GPRK5 has been shown to serve as a substrate for PKC, although PKC-mediated phosphorylation inhibits GPRK5 (Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Krasel, C *et al. J Biol Chem*, 2001, 276: 1911-1915). In addition, the extreme N terminus of GPRK5 contains a binding site for Ca2+/calmodulin, where upon binding it inhibits GPRK activity, a mechanism suggested to regulate GPRKs activity (Krasel, C *et al. J Biol Chem*, 2001, 276: 1911-1915). Since GnRH receptors are a member of the G-protein coupled receptor (GPCR) family and recruit and activate the components of several signaling pathways, including PKC and Ca2+/calmodulin, their regulatory interaction with GPRKs may serve in regulating various events downstream from these signals in LSMC and MSMC.

Nuclear translocation of many activated intracellular signaling molecules results in phosphorylation and activation of transcription factors, major elements in these signaling networks that regulate specific gene expression. In previous studies (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71) and the present study, several transcription factors were identified as differentially expressed and regulated in leiomyoma and myometrium and targeted by GnRHa direct action in LSMC and MSMC (see Tables 1-4). Many of these transcription factors are involved in ovarian steroids, polypeptide hormones, inflammatory cytokines, growth factors and ECM receptor mediated-actions, by regulating the promoter of their target genes in various normal and cancer cells. However, little is known regarding the expression and regulation of these and other transcription factors in leiomyoma and myometrium. For this reason, the present inventors placed a greater emphasis on verification of the expression of transcription factors such as Nur77, CITED2, EGR3, TIEG and TGIF in leiomyoma, myometrium and their temporal regulation by GnRHa in LSMC and MSMC.

Nur77 (also known as NR4A1, TR3, NGFI-B, NAK-1) is a member of the orphan nuclear receptor superfamily originally identified as an immediate-early gene in serum-treated fibroblasts (Maira, M *et al. Mol and Cell Biol*, 2003, 23;763-776; Drouin, J *et al. J. Steroid Biochem Mol Biol*, 1998, 65:59-63; Fernandez, P *et al. Endocrinology*, 2000, 141:2392-2400; Gelman, L *et al. J Biol Chem*, 1999, 274:7681-7688; Sadie, H *et al. Endocrinology*, 2003,

144:1958-71; Wilson, TE *et al. Mol Cell Biol*, 1993, 13:861-868; Song, KH *et al. Endocrinology*, 2001, 142:5116-23; Zhang, P and Mellon, SH *Mol Endocrinol*, 1997, 11:891-904). It is also identified as NGF-inducible gene, which is constitutively expressed in various tissues and is strongly induced by several stimuli, resulting in regulation of gene expression related to inflammation, angiogenesis, apoptosis and steriodogenesis, including steroid-21 and 17α-hydroxylases and 20α hydroxysteroid dehydrogenase in the hypothalamic-pituitary-adrenal axis (Maira, M *et al. Mol and Cell Biol*, 2003, 23;763-776; Drouin, J *et al. J. Steroid Biochem Mol Biol*, 1998, 65:59-63; Fernandez, P *et al. Endocrinology*, 2000, 141:2392-2400; Gelman, L *et al. J Biol Chem*, 1999, 274:7681-7688; Sadie, H *et al. Endocrinology*, 2003, 144:1958-71; Wilson, TE *et al. Mol Cell Biol*, 1993, 13:861-868; Song, KH *et al. Endocrinology*, 2001, 142:5116-23; Zhang, P and Mellon, SH *Mol Endocrinol*, 1997, 11:891-904). In the anterior pituitary, Nur77 is reported to mediate the stimulatory effect of CRH and the negative-feedback regulation of POMC transcription by glucocorticoids, as well as GnRH-induced GnRH receptor expression (Drouin, J *et al. J. Steroid Biochem Mol Biol*, 1998, 65:59-63; Sadie, H *et al. Endocrinology*, 2003, 144:1958-71). LH-induced Nur77 is also reported to regulate cytochorome p450 expression in granulosa and leydig cells (Sadie, H *et al. Endocrinology*, 2003, 144:1958-71; Wilson, TE *et al. Mol Cell Biol*, 1993, 13:861-868; Song, KH *et al. Endocrinology*, 2001, 142:5116-23). More importantly, overexpression of Nur77 is implicated as an important regulator of apoptosis in different cells. In response to apoptotic stimuli, Nur77 translocation from the nucleus to mitochondria results in cytochrome C release and apoptosis of LNCaP human prostate cancer cells (Rajpal, A *et al. EMBO J*, 2003, 22:6526-36; Castro-Obregon, S *et al. J Biol Chem*, 2004, 279:17543-53; Li, H *et al. Science*, 2000, 289:1159-1164). The present inventors found a relatively similar expression of Nur77 in myometrium and leiomyoma; however, GnRHa therapy resulted in a significant elevation of Nur77 in both tissues. GnRHa treatment also resulted in a rapid induction of Nur77 in MSMC and LSMC, which subsequently declined to control levels, and in LSMC fell to below the levels detected in untreated cells. Interestingly, GnRH is reported to regulate Nur77 expression in αT3-1 and LβT2 gonadotrope cell lines through PKA pathway and GnRH receptor promoter via a mechanism involving SF-1 with Nur77 acting as a negative regulator of this response (Sadie, H *et al.*

Endocrinology, 2003, 144:1958-71). In a recent study, activation of MAPK pathway involving Raf-1, MEK2 and ERK2 was reported to regulate Nur77 activation resulting in nonapoptotic program cell death (Castro-Obregon, S *et al. J Biol Chem*, 2004). The present inventors have shown that GnRH signaling through MAPK and transcriptional activation of c-fos and c-jun regulate the expression of several specific genes in LSMC and MSMC. This suggests that GnRH-mediated action through this pathway may regulate nur77 expression thus influencing the outcome of cellular growth arrest and/or apoptosis in leiomyoma.

Recently, a new family of transcriptional co-regulators, the CITED (CBP/p300-interacting transactivator with ED-rich tail) family, was discovered that interact with the first cysteine-histidine-rich region of CBP/p300 (Tien, ES *et al. J Biol Chem*, 2004, 279:24053-63; Kranc, KR *et al. Mol Cell Biol*, 2003, 23:7658-66). The CITED family contains four members and appears to act as key transcriptional modulators in embryogenesis, inflammation, and stress responses (Tien, ES *et al. J Biol Chem*, 2004, 279:24053-63) by affecting the transcriptional activity of many transcription factors ranging from AP2, estrogen receptor, and hypoxia-inducible factor 1 (HIF1) and LIM (Yin, Z *et al. Proc Natl Acad Sci USA*, 2002, 99:10488-10493). The present inventors identified CITED2 among the differentially expressed and regulated genes in leiomyoma, myometrium and their isolated cells, and in response to GnRHa treatment *in vivo* and *in vitro*. Unlike GnRHa therapy which increased CITED2 expression in leiomyoma and myometrium, GnRHa had a biphasic effect on CITED2 expression in MSMC, while inhibiting expression in LSMC. Although *in vitro* culture conditions may directly influence the expression of regulatory molecules that either interact with or regulate CITED2 expression, the exact molecular mechanism resulting in differential expression of CITED2 *in vivo* and *in vitro* by GnRHa requires further investigation. Interestingly, the expression of several growth factors, cytokines and HIF1 are the target of ER, PR regulatory action, and CITED2 acting as a repressor of their expression may serve as an important regulator of processes that regulate inflammatory response, angiogenesis and tissue remodeling in leiomyoma. Additionally, CBP/p300 which serve as promiscuous co-activators for an increasing number of transcription factors resulting in proliferation, differentiation and apoptosis in response to diverse biological factors, including ER- and PR-dependent transcriptional activity,

is specifically recruited by Nur77 acting as dimers following PKA activation (Maira, M *et al. Mol and Cell Biol*, 2003, 23;763-776; Kranc, K *et al. Trends Cell Biol*, 1997, 7:230-236; Puri, PL *et al. EMBO J*, 1997, 16:369-383).

In a previous microarray study, it was reported that EGR1, a prototype of a family of zinc-finger transcription factors that includes EGR2, EGR3, EGR4, and NGFI-B (Hjoberg, J *et al. Am J Physiol Lung Cell Mol Physiol*, 2004, 286:L817-825; Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92), is differentially expressed in leiomyoma and myometrium (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71). Here, the present inventors provide evidence for the expression of EGR3 and differential regulation in response to GnRHa therapy in leiomyoma and myometrium, as well as in LSMC and MSMC *in vitro*. A recent report demonstrated that EGR1 expression is elevated in leiomyoma compared to corresponding myometrium in women who received GnRHa therapy (Shozu, M *et al. Cancer Research*, 2004, 64:4677-4684) supporting previous microarray data (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71). EGRs expression is rapidly and transiently induced by a large number of growth factors, cytokines, polypeptide hormones and injurious stimuli and kinetics of their expression is essentially identical to c-fos proto-oncogene (Hjoberg, J *et al. Am J Physiol Lung Cell Mol Physiol*, 2004, 286:L817-825; Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92; Inoue, A *et al. J Mol Endocrinol*, 2004, 32:649-61). In addition, induction of EGR1 occurs primarily at the level of transcription and is mediated, in part, through MAPKs, including ERK, JNK, and p38 pathways (Hjoberg, J *et al. Am J Physiol Lung Cell Mol Physiol*, 2004, 286:L817-825; Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92). It has been demonstrated that GnRHa through the activation of MAPK regulates the expression c-fos and c-jun as well as fibronectin, collagen and PAI-1 expression (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)). In human fibrosarcoma and glioblastoma cells, EGR directly influences the expression of fibronectin, TGF-β1, and PAI-1 and may regulate the expression of PDGF, tissue factor, and membrane type 1 matrix metalloproteinase (Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92; Liu, C *et al. J Biol Chem*, 1999, 274:4400-11). Estrogen is also reported to induce EGR3 in various cancer cell lines while is inhibited by progesterone in Schwann cells (Inoue, A *et al. J Mol Endocrinol*, 2004, 32:649-61; Mercier, G *et al. Mol Brain Res*, 2001, 97:137-148). Constitutive transgenic

expression of EGR3 has recently been shown to increase thymocytes apoptosis, possibly through potent activation of FasL expression (Xi, H and Kersh, GJ *J Immunol*, 2004, 173:340-8). Given the role of ovarian steroids and a large number of growth factors, cytokines and polypeptide hormones in leiomyoma growth, and suppression by GnRHa, their differential influence on EGR1 and EGR3 expression may represent a mechanism resulting in balance between the rate of cell proliferation and apoptosis as well as tissue turnover, affecting leiomyoma growth and regression.

The present study also provides the first evidence of the expression and regulation of TIEG and TGIF, novel three zinc-finger Kruppel-like transcriptional repressors, and key regulators of TGF-β receptor signaling (Johnsen, SA *et al. Oncogene*, 2002, 21:5783-90; Cook, T and Urrutia, R *Am J Physiol Gastrointest Liver Physiol*, 2000, 278:G513-21; Ribeiro, A *et al. Hepatology*, 1999, 30:1490-7; Chen, F *et al. Biochem J*, 2003, 371:257-63; Melhuish, TA *et al. J Biol Chem*, 2001, 276:32109-14), by GnRHa in leiomyoma, myometrium, LSMC and MSMC. TIEG regulates TGF-β receptor signaling through a negative feedback mechanism by repressing the inhibitory Smad7 (Johnsen, SA *et al. Oncogene*, 2002, 21:5783-90). In addition, TGIF through direct binding to DNA or interaction with TGF-β-activated Smads represses TGF-β-responsive gene expression (Chen, F *et al. Biochem J*, 2003, 371:257-63; Melhuish, TA *et al. J Biol Chem*, 2001, 276:32109-14). Since GnRHa suppresses TGF-β and TGF-β receptors while enhancing Smad7 expression in leiomyoma and myometrium as well as LSMC and MSMC, differential regulation of TIEG and TGIF may serve as an additional downstream mechanism altering TGF-β autocrine/paracrine actions in leiomyoma. To further understand the regulation of these transcription factors in leiomyoma, the present inventors also provide evidence for their regulation in LSMC and MSMC by TGF-β, further implicating the importance of TGF-β in pathogenesis of leiomyoma (as described in Examples 4-7).

The expression, activation and direct interaction of these and other transcription factors with DNA results in regulation of the expression of various genes whose products influence cell growth, inflammation, angiogenesis, apoptosis and tissue turnover. In previous studies (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)) and the present study, several differentially expressed and regulated genes were identified in leiomyoma, myometrium and LSMC and MSMC whose promoters are the target of

these transcription factors. Among these genes are members of cell cycle regulatory proteins that play a central role in leiomyoma growth and regression (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50), including p27, p57 and Gas1. The present inventors identified p27, p57 and Gas1 as differentially expressed and regulated in leiomyoma and myometrium as well as LSMC and MSMC and in response to GnRHa treatment. Although p27, p57 and Gas1 function as major regulators of cell cycle progression, several studies have also shown Cip/Kip proteins function as transcriptional cofactors by regulating the activity of NFκ-B, STAT3, Myc, Rb, C/EBP, CBP/p300, E2F and AP1 (Coqueret, O *Trends Cell Biol*, 2003, 13:65-70). A recent report also suggests that p21, p27 and p57 are involved in regulation of apoptosis (Blagosklonny, MV *Semin Cancer Biol*, 2003, 13:97-105) and their differential regulation in leiomyoma and myometrium is consistent with GnRHa induction of apoptosis related gene in LSMC and MSMC (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50). However, the results disclosed herein are the first to document the expression of Gas1 in leiomyoma and myometrium, and regulation in LSMC and MSMC in response to timed-dependent action of GnRHa. GnRHa has been demonstrated to alter cell cycle progression and programmed cell death in several cell types including leiomyoma smooth muscle cells (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50), and these results provide additional support for the involvement of specific cell cycle and apoptotic related genes in leiomyoma growth and regression. How the expression of these genes is regulated and through what mechanism their products influence LSMC and MSMC cell cycle progression and programmed cell death awaits further investigation.

Leiomyoma growth and GnRHa therapy resulting in leiomyoma regression also involves extracellular matrix turnover. In previous studies(Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71), in the present study, and in recent studies by other groups (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108), several genes in this category were identified displaying differential expression in leiomyoma and myometrium and were targeted by GnRH therapy (Tables 1-4) (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Dou, Q *et al. Mol Hum Reprod*, 1997, 3:1005-1014; Luo, X *et al.* 2004 Gene expression profiling of leiomyoma and myometrial smooth muscle cells in response to TGF-b autocrine/paracrine action. Accompanying paper; Levens, E *et al. Fertil Steril*, 2004, (In press); Stewart, EA *et al. J Clin Endocrinol Metab*, 1994, 79:900-6). These include the expression of several collagens, small leucine rich repeat family of proteoglycans, decorin, biglycan, osteomodulin, fibromodulin, versican, and osteoadherin/osteoglycin, fibronectin, desmin and vimentin, several member of proteases such as matrix metalloproteinases (MMPs) and their inhibitors, TIMPs, a disintegrin-like and metalloproteinase proteins (ADAM), *etc.* It has also been reported that GnRHa regulates the expression of fibronectin, collagen type I, PAI-I, MMPs and TIMPs (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press); Dou, Q *et al. Mol Hum Reprod*, 1997, 3:1005-1014), as well as decorin, versican, desmin and vimentin (unpublished data) in leiomyoma and myometrium, involving the activation of MAPK in LSMC and MSMC (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:(in press)). Since ECM turnover is a key regulator of the outcome of tissue fibrosis, and many cytokines, chemokines, growth factors and polypeptide hormones through specific intracellular signal transduction and activation of transcription factors influence the expression of ECM and proteases, further investigation is underway to elucidate their regulatory interactions affecting processes that may influence leiomyoma growth and regression.

In summary, in the present study, the inventors provide a comprehensive assessment of the gene expression profile of leiomyoma and matched myometrium during early-mid luteal phase of the menstrual cycle, a period characterized by elevated production of ovarian steroids and maximal leiomyoma growth, compared with tissues obtained from hormonally suppressed patients on GnRHa therapy and in response to the direct action of GnRHa on LSMC and MSMC. The present inventors identified several common and tissue-specific gene clusters in these cohorts suggesting their co-regulation by the same factors and or mechanism(s) in the same cluster. The present inventors validated the expression of several genes whose products are important in signal transduction, transcription, cell cycle regulation, apoptosis and ECM turnover, events critical to development, growth and regression of leiomyoma. Based on these and previous observations, the present inventors propose that the product of these specific genes, by regulating the local inflammatory and apoptotic processes leading to elaboration of profibrotic cytokines production such as TGF-β is central to the establishment and progression of fibrosis in leiomyoma. Provided in Examples 4-7 is further evidence for the role of TGF-β autocrine/paracrine action in this process.

Example 4—Gene Expression Profiles of Leiomyoma and Matched Myometrium Cells In Response to TGF-β1

It has been reported that leiomyoma and myometrium express all the components of the TGF-β system, and it has been shown that TGF-β through Smads and MAPK pathways regulates the expression of a specific number of genes in LSMC and MSMC (7-12). Here, the present inventors performed microarray analysis to further characterize the molecular environment of LSMC and MSMC directed by TGF-β autocrine/paracrine actions. Using the same cell preparations and culture conditions described in the accompanying manuscript (22), LSMC and MSMC were treated with TGF-β1 (2.5 ng/ml) for 2, 6 and 12 hrs, total RNA was isolated and subjected to microarray analysis. Following global normalization and transformation of the gene expression values, supervised learning, discrimination analysis, cross validation and variation filtering, the gene expression values for this study were independently subjected to statistical R programming analysis and ANOVA with false discovery rate selected at $p \leq 0.001$. The analysis identified 310

genes or 2.46% of the genes on the array as differentially expressed and regulated in response to time-dependent action of TGF-β in LSMC and MSMC.

As illustrated in Figure 12, hierarchical clustering analysis separated these differentially expressed genes into distinctive clusters, with sufficient difference in their patterns allowing each cohort to cluster into their respective subgroup. The differentially expressed and regulated genes were separated into five clusters in response to time-dependent action of TGF-β in LSMC and MSMC, with genes in clusters A and B displaying a late response, genes in cluster D displaying early response, and genes in clusters C and E showing biphasic regulatory behaviors (Figure 12). Further analysis of the variance-normalized mean gene expression values divided the genes into 6 clusters, each displaying a different level of response to time-dependent action of TGF-β, with overlapping behavior between LSMC and MSMC with the exception of genes in clusters E and F (Figure 13)

Comparative analysis between gene expression profiles of LSMC and MSMC in response to TGF-β action, with their corresponding leiomyoma and myometrium (tissues) from the untreated group (22) revealed a substantial variability among their profiles (data not shown). However, gene ontology assessment and division into functional categories indicated that the majority of these genes (60 to 70%) are involved in transcriptional regulation and metabolism, cell cycle regulation, extracellular matrix and adhesion molecules, signal transduction and transcription factors (ref.#22, Figure 14). The time-dependent action of TGF-β on expression the profile of a selective group of these genes in the above clusters representing transcription factors, growth factors, cytokines, signal transduction pathways, ECM/adhesion molecules *etc.* in LSMC and MSMC are presented in Figure 15.

<u>Example 5—Gene Expression Profiles of LSMC and MSMC In Response to TGF-β Following Pretreatment with TGF-β type II Receptor Antisense</u>

To further evaluate the autocrine/paracrine action of TGF-β in leiomyoma and myometrial microenvironments, LSMC and MSMC were pretreated with TGF-β type II receptor (TGF-β type IIR) antisense oligomers to block/reduce TGF-β receptor signaling. Following pretreatments the cells were treated with or without TGF-β for 2 hrs and their total RNA was subjected to microarray

analysis. Based on the same data analysis described above with false discovery rate of $p \leq 0.001$, the present inventors identified 54 differentially expressed and regulated genes in response to TGF-β1 (2.5 ng/ml for 2 hrs) in LSMC and MSMC pretreated with TGF-β type IIR antisense. Hierarchical cluster analysis distinctively separated these genes into 3 clusters with each cohort separated into their respective subgroups (Figure 16). The genes in clusters A and C displayed different response to TGF-β type IIR antisense treatment, while genes in cluster B showed overlapping behavior in LSMC and MSMC (Figure 16). However, there was an overlapping pattern between the gene expression profiles in TGF-β type IIR sense- and antisense-treated cells that could be due to the inability of antisense treatment to block all the combined action of autocrine/paracrine and exogenously added TGF-β. Interestingly, antisense treatment altered the expression of many genes known to be the target of TGF-β action, including those validated in this study. Gene ontology assessment and division into similar functional categories indicated that the majority of these genes are involved in transcriptional regulation and metabolism, cell cycle regulation, extracellular matrix and adhesion molecules, and transcription factors (Figure 14).

Example 6—Comparative Analysis of Gene Expression Profiles in Response to TGF-β type II Receptor Antisense and GnRHa Treatments In LSMC and MSMC

Since GnRHa alters the expression of TGF-β and TGF-β receptors expression in leiomyoma and myometrium as well as in LSMC and MSMC, the present inventors compared the gene expression profile of TGF-β type IIR antisense-treated with GnRHa-treated LSMC and MSMC, searching for common genes whose expression are affected by these treatments. Based on the same data analysis described above with false discovery rate selected at $p \leq 0.001$, the present inventors identified 222 genes differentially expressed and regulated in LSMC and MSMC in response to TGF-β type IIR antisense- and GnRHa-treated cells (Tables 7 and 8). Hierarchical clustering analysis separated these genes into 4 clusters displaying different pattern of regulation allowing their separation into respective subgroup (Figure 17). The genes in cluster A, B and D displayed different response to TGF-β type IIR antisense and GnRHa treatments, with genes in cluster C showing overlapping behavior in LSMC and MSMC (Figure 17).

Table 7. Categorical list of genes identified as differentially expressed in LSMC pretreated with TGF-β type II receptor (TGF-β type IIR) antisense for 24 hrs followed by TGF-β treatment for 2 hrs compared to LSMC treated with GnRHa (0.01 μM) for 2, 6, 12 hrs (data derived from the experiments described in the accompanying manuscript; see reference #22). The genes were identified following supervised analysis of their expression values and statistical analysis in R programming and ANOVA with a false-discovery rate of rate of $p \leq 0.001$.

Table 8. Categorical list of genes identified as differentially expressed in LSMC pretreated with TGF-b type II receptor (TGF-b type IIR) antisense for 24 hrs followed by TGF-b treatment for 2 hrs compared to LSMC treated with GnRHa (0.01 μM) for 2, 6, 12 hrs (data derived from the experiments described in the accompanying manuscript). The genes were identified following supervised analysis of their expression values and statistical analysis in R programming and ANOVA with a false-discovery rate of rate of $p \leq 0.001$

<u>Example 7—Verification of Gene Transcripts in TGF-β-treated LSMC and MSMC</u>

Using Realtime PCR, the present inventors validated the expression of 12 genes in response to time dependent action of TGF-β in LSMC and MSMC (Figure 18). They include IL-11, CITED2, Nur77, EGR3, TIEG, TGIF, p27, p57, GAS-1 and GPRK5, whose expression was also validated in leiomyoma and matched myometrium from untreated and GnRHa-treated cohorts as well as LSMC and MSMC treated in vitro with GnRHa as described in the accompanying manuscript (22). In addition, the present inventors verified the expression of Runx1 and Runx2. As illustrated TGF-β in a time dependent manner differentially regulate the expression of these genes in LSMC and MSMC with a pattern of expression displaying significant overlap between Realtime PCR and microarray analysis (Figure 18). However, the expression value of GPRK5 and Runx2 genes in microarray analysis of LSMC and MSMC did not meet the standard of analysis and was not included among the list of differentially expressed and regulated genes in response to TGF-β, although Runx2 mRNA is detectable by Realtime PCR (Figure 18). The results indicated that Runx1 and Runx2 expression not only is the target of TGF-β regulatory action, they are also regulated by GnRHa therapy in leiomyoma and

myometrium as well as by GnRHa in LSMC and MSMC *in vitro*, with their time-dependent inhibition in MSMC (Figure 18).

The present inventors verified the expression of IL-11, TIGF, TIEG, p27 and p57 by Western blotting and their cellular distribution using immunohistochemistry in leiomyoma and myometrium (22). These findings provide further support for the microarray and Realtime PCR data indicating that the products of these genes are expressed in leiomyoma and myometrium. The present inventors are currently investigating time-dependent and dose-dependent regulation of these genes in response to TGF-β.

By extending previous work on the role of TGF-β in leiomyoma, in this study, the present inventors have provided the first example of gene expression fingerprints of LSMC and MSMC in response to autocrine/paracrine action of TGF-β. The present inventors further characterized the molecular environment of these cells following pretreatment with TGF-β type IIR antisense as a tool to interfere with the autocrine/paracrine action of TGF-β isoforms, and comparatively assessed their expression profiles with GnRHa-treated cells, which also inhibits TGF-β receptor expression in these cells (6,8). Since the aim of this study was to capture the early and late autocrine/paracrine action of TGF-β in these cells, the present inventors selected a treatment strategy based on previous observations reflecting TGF-β time-dependent regulation of c-fos, c-jun, fibronectin, collagen type I, and PAI-1 expression (11). TGF-β regulates the expression of these genes in LSMC and MSMC through TGF-β receptor activation of Smad and MAPK pathways (3,10,11), whose promoters are known to contain TGF-β regulatory elements (23,24). This study design is also consistent with other microarry studies profiling gene expression in response to TGF-β action in human dermal fibroblasts, HaCaT kritonocyte cell line and NMuMG, mouse mammary gland epithelial cell line, in which the cells were treated for 1, 2, 6 and 24 hrs, displaying a Smad-mediated regulation of selected number of genes (25-27).

Cluster and tree-view analysis revealed a considerable similarity in overall gene expression patterns between LSMC and MSMC in response to TGF-βaction; however, there was sufficient difference allowing their separation into respective subgroups. The genes in these clusters displayed different regulatory response to TGF-β action in a cell- and time-specific manner, with genes in clusters A and B displaying a late response, genes in cluster D displaying early

responsiveness, and clusters C and E showing a biphasic regulatory behavior. These results suggest that the same factors and/or mechanisms co-regulate the expression of these genes in each cluster, possibly due to the presence of common regulatory elements in their promoters. Whether the expression profile of these genes in LSMC and MSMC respond differently to varying concentration of TGF-β, or other TGF-β isoforms is not established. However, the concentration of TGF-β used in this and other studies examining the effect of TGF-βon the expression of other genes (10-13,25-27), is comparable with level of TGF-β produced by these cells, although LSMC produces more TGF-β1 compared to MSMC (7,8). Moreover, based on the profile of TGF-β isoforms's expression in leiomyoma, it has previously been proposed that TGF-β1 and TGF-β3 play an more critical role in leiomyoma (7), and *in vitro* studies have indicated a higher growth response to TGF-β1 (personal observations) and TGF-β3 in LSMC compared to MSMC (14,15). However, TGF-β isoforms mediate their actions through TGF-β type IIR, and alterations in the TGF-β receptor system may serve as a more accurate indicator of their overall autocrine/paracrine actions in these and other cell types. It has been shown that leiomyoma over-expresses TGF-β type IIR compared to myometrium (6,9), and pretreatment of LSMC with TGF-β type IIR antisense oligomers and/or neutralizing antibodies prevented TGF-β receptor-mediated actions (8,10).

These observations as well as identification of specific genes whose expression exhibited sensitivity to pretreatment with TGF-β type IIR antisense, among them genes containing TGF-β regulatory response elements in their promoters, further support TGF-β receptors mediated signaling in regulating the overall expression of these genes in LSMC and MSMC, and possibly in leiomyoma and myometrium. Lack of response of other TGF-β-targeted genes to TGF-β type IIR antisense pretreatment could be due to inability of antisense to block all the autocrine/paracrine, as well as exogenously added TGF-$\tilde{\beta}$. However, the expression of these genes may also be regulated as a consequence of TGF-β receptors overexpression and/or their altered intracellular signaling. Interestingly, activin receptor-like kinases (ALK) ALK1 and ALK5, which serve as TGF-β type I receptors and are activated by TGF-β type II receptors, have been shown to regulate the expression of different genes in endothelial cell in response to TGF-β action (28). However, ALK1 functions as a TGF-β type I receptor in endothelial cells, while ALK-5 is expressed in various cells, and

through distinct Smad proteins, *i.e.*, Smadl/Smad5 and Smad2/Smad3, respectively, regulate gene expression in response to TGF-β actions (28). The present inventors have identified the expression of all the components of the TGF-β receptor system, including ALK5 and Smad2/3 in leiomyoma and myometrium as well as LSMC and MSMC. However, TGF-β-mediated action through ALK1 could result in the regulation of a different set of genes not involving ALK5. In addition to TGF-β and TGF-β receptors, alteration in Smad expression is also considered to influence the outcome of several disorders targeted by TGF-β including tissue fibrosis (2).

Gene ontology dividing the differentially expressed and regulated genes into similar functional categories revealed that the majority of the genes targeted in response to TGF-β treatment of LSMC and MSMC are associated with cellular metabolism, cell growth regulation (cell cycle and apoptosis), cell and tissue structure (ECM, adhesion molecules and microfilements), signal transduction and transcription factors. Despite the differences in their profiles, the present inventors found a substantial degree of similarity in functional annotation among the genes identified at tissue (leiomyoma and myometrium, Ref#22) and cellular (LSMC and MSMC) levels in response to TGF-β1. These differences between gene expression profiles of tissues and LSMC/MSMC in response to TGF-β could be due to the contribution of other cell types to the gene pool, and the influence of other autocrine/paracrine regulators on the overall genes expression at the tissue level. Previous studies from this laboratory and others have reported the expression of a few other genes targeted by TGF-β action in LSMC and MSMC. However, to the present inventors' knowledge, this is the first example of a large-scale gene expression profiling of these cells in response to TGF-β. Using quantitative realtime PCR analysis, the presenti inventors validated the expression of several of these genes in response to time-dependent action of TGF-β in LSMC and MSMC, including the expression of 10 genes validated in leiomyoma/myometrium as well as in LSMC/MSMC in response to GnRHa treatment (22).

The present inventors demonstrated that LSMC express an elevated level of IL-11 compared to MSMC, and its expression is a major target of TGF-β regulatory action. Although the biological significance of IL-11 expression in leiomyoma and myometrial environments, and consequence of its overexpression in leiomyoma await investigation, IL-11, alone, or through

interaction with TGF-β, is considered to play a critical role in progression of fibrotic disorders (29-32). Other members of the interleukin family, IL-4 and IL-13, and their interactions with TGF-β are also reported to be equally important in this disorder (33,34). IL-13 expression has recently been identified in leiomyoma, and it has been discovered that exposure of LSMC to IL-13 upregulates the expression of TGF-β and TGF-β type II receptors in LSMC, suggesting a direct, and/or indirect regulatory function for IL-13 in mediating events leading to progression of tissue fibrosis in leiomyoma (19). Other cytokines in this category including IL-4, IL-6, IL-8, IL-15, IL-17, TNF-□ and GM-CSF are also expressed in leiomyoma and myometrium (19-21). These cytokines are classified as type1/type2 related subsets and predominance toward type II direction is considered to result in inflammatory/immune responses leading to progression of tissue fibrosis (31-35). A recent report has further elaborated the participation of IL-11 and TGF-β, and transcription factor EGR1 in tissue fibrosis, through a mechanism involving regulation of the balance between the rate of cellular apoptosis and inflammatory response (35). EGR1 has previously been identified among the differentially expressed genes in leiomyoma and myometrium (21) and expression of EGR2 and EGR3 in these tissues (22) and regulation of EGR3 in response to TGF-βaction in LSMC and MSMC is demonstrated herein.

Elevated expression and preferential phosphorylation of EGR1 leads to regulation of target genes whose products are involved in apoptosis as well as angiogenesis and cell survival, including IL-2, TNF-alpha, Flt-1, Fas, Fas ligand, cyclin D1, p15, p21, p53, PDGF-A, angiotensin II-dependent activation of PDGF and TGF-β, VEGF, tissue factor, 5-lipoxygenase, thymidine kinase, superoxide dismutase, intercellular adhesion molecule 1 (ICAM-1), fibronectin, urokinase-type plasminogen activator and matrix metalloproteinase type 1 (36-41). The expression of many of these genes has been documented in myometrium and leiomyoma (1), and known to be the target of TGF-βregulatory action. EGR1 also acts as a transcriptional repressor of TGF-β type II receptor through direct interaction with SP1 and Ets-like ERT sites in proximal promoter of the gene (41). Transfection of EGR1 expression vector into a myometrial cell line (KW) expressing low levels of EGR1 is reported to result in a rapid growth inhibition of these cells (42). To the present inventors' knowledge, this is the first report of the regulatory action of TGF-β on EGR3 expression, not only in LSMC and MSMC, but any other cell types.

Based on previous and present observations, the present inventors propose that a local inflammatory response mediated through individual and combined actions of TGF-β, IL-13 and IL-11, as well as regulatory function of TGF-β on EGR expression, results on local expression of set of genes whose products promote apoptotic and non-apoptotic cell death, further enhancing an inflammatory reaction that orchestrate various events leading to progression of fibrosis in leiomyoma.

Additional genes identified as differentially expressed and regulated by TGF-β autocrine/paracrine action in LSMC and MSMC in this functional category include TGIF, TIEG, CITED2, Nur77, Runx1 and Runx2. These transcription factors possess key regulatory functions in the expression of a wide range of genes in response to various stimuli specifically TGF-β. The expression of TGIF, TIEG, CITED2 and Nur77 is highly regulated in LSMC and MSMC, and with the exception of CITED2, TGF-β transiently increased their expression in a time-dependent manner. TGIF is a transcriptional co-repressor that directly associates with Smads and inhibits Smad-mediated transcriptional activation by competing with p300 for Smad association (43,44). CITED2, induced by multiple cytokines, growth factors and hypoxia, also interacts with p300 and function as a coactivator for transcription factor AP-2 (45). CITED2-mediated action is reported to result in down-regulation of MMP-1 and MMP-13 through interactions with CBP/p300 and other transcription factors such as c-fos, Ets-1, NF□B, and Smads that control MMPs promoter activities (46,47). TGF-β targets the expression of these transcription factors and MMPs in many cell types, including LSMC and MSMC (11,47,48), thus their differential regulation and interactions with CITED2 and TGIF may serve in regulating the outcome of TGF-β autocrine/paracrine actions in leiomyoma involving cell growth, inflammation, apoptosis and tissue turnover. Unlike TGIF, TIEG is rapidly induced by TGF-β and enhances TGF-β actions through Smad2/3 activation (49-51). However, TIEG has no effect on gene transcription in the absence of Smad4, or due to overexpression of Smad7, although it is capable of increasing Smad2/3 activity in the absence of Smad7 (47,49). It was shown that TGF-β induced a rapid, but transient expression of TIEG in LSMC and MSMC, and the expression of Smad2/3, Smad4 and Smad7 and their differential regulation by TGF-β has been demonstrated in these cells (10,11). Based on these observations, the present inventors further propose that TGF-β through a

mechanism involving TGIF, TIEG and Smads self regulates its own autocrine/paracrine action in leiomyoma/ myometrium. Estrogen has also been shown to increase TIEG expression in breast tumor cell (49,52). Since estrogen, a major growth-promoting factor for leiomyoma, induces TGF-β expression in LSMC and MSMC (7,8), E2-induced TGF-β or estrogen directly may regulate TIEG expression in leiomyoma. TIEG is also reported to trigger apoptotic cell programs by a mechanism involving the formation of reactive oxygen species (51), often created as a result of local inflammatory response. Whether TGF-β-induced TIEG through the above mechanism results in apoptotic response in leiomyoma is not known; however, formation of reactive oxygen species may enhance local inflammatory response serving as an additional mediator of tissue fibrosis in leiomyoma.

With respect to Nur77, it regulates the expression of a group of genes whose products are involved in cell cycle regulation, differentiation, apoptosis, and malignant transformation (53,54). Evidence has been provided that Nur77 is the target of regulatory action of TGF-βin LSMC and MSMC, with pattern of expression resembling that observed in leiomyoma and myometrium, respectively (21,22). Although the nature and functional significance of Nur77 expression in leiomyoma, and regulation by TGF-β is unknown, malignant transformation in leiomyoma is rare, suggesting Nur77 may function as regulator of cell cycle in leiomyoma and myometrium. In addition to Nur77, the present inventors discovered that the expression of various genes functionally associated with cell cycle regulation and apoptosis are influenced by TGF-β autocrine/paracrine action, and balance of their expression may become a critical factor in leiomyoma growth and regression. Additional transcription factors whose expression was the target of TGF-β action in LSMC and MSMC are Runx1 and Runx2. This family of transcriptional factors consisting of Runx1 to Runx3, are integral components of signaling cascades mediated by TGF-β and bone morphogenetic proteins regulating various biological processes, including cell growth and differentiation, hematopoiesis and angiogenesis (23,47,55-57). The present inventors provided the first evidence for regulatory action of GnRHa therapy and GnRHa direct action on Runx1 and Runx2 expression in leiomyoma, myometrium as well as LSMC and MSMC, with GnRHa significantly inhibiting their expression, specifically in MSMC. Although Runx2 is expressed at low levels in leiomyoma and myometrium, Runx1 and Runx2

expression in LSMC and MSMC displayed a rapid response to TGF-β action *in vitro*, with Runx1 showing a significantly higher response. TGF-β activation of Smad and MAPK cascades regulates the expression of Runx2; however, interaction with Smad3 causes repression of Runx2 and downstream transcription activation of specific genes (23,47,57). It has recently been reported that TGF-β and GnRH activate the MAPK pathway (11), and GnRHa alter TGF-β-activated Smad in LSMC and MSMC (10), signaling cascade that may regulate Runx1 and Runx2 expression in these cells. Differential regulation of Runx1 and Runx2 by TGF-β and GnRHa imply their potential biological implication, specifically in regulating TGF-β action in leiomyoma microenvironment. This is particularly interesting since estrogen is also reported to enhance Runx2 activity, through a mechanism involving TGF-β type I receptor gene promoter, which contains several Runx binding sequences (56). Together, the identification of these and several other key transcription factors in LSMC and LSMC, and their regulation by TGF-β serving as integral components of inflammatory, cell cycle and apoptotic processes, further support the present inventors' hypothesis that a regulatory balance between these events is a key factor in progression of fibrosis mediated by TGF-β in leiomyoma.

Such balance between cell proliferation and apoptosis is critical to tissue homeostasis and central to leiomyoma growth and regression. Since both positive and negative signals determine the outcome of these events, the present inventors searched and identified several genes in this category in previous studies and in the current study as differentially expressed and regulated in leiomyoma and myometrium, as well as in LSMC and MSMC in response to TGF-β. The primary focus here was placed on p27Kip1, p57Kip2 and Gas1 expression, because of their regulation by GnRHa as demonstrated in the accompanying manuscript (22). It was found that TGF-β suppressed the expression of these genes in LSMC, and in a biphasic fashion accompanied by suppression of GAS1 expression in MSMC. TGF-β is known to regulate the expression of several cell cycle regulatory proteins including p27, which bind cyclin-dependent kinase (CDK), and by inhibiting catalytic activity of CDK-cyclin complex, regulate cell cycle progression and apoptosis (58). However, TGF-β regulation of p57 expression is limited (23,24,59) and available data suggests that TGF-β enhances p57 degradation through ubiquitin-proteasome pathway and Smad-mediated signaling (60). TGF-β-induced p57 degradation,

CDK2 activation and cell proliferation is blocked by proteasome inhibitors and/or by overexpression of Smad7 (60-63). TGF-β-induced cell growth is also influenced by c-myc and the expression and activities of G1, G2, CDK and cyclins, and their inhibitors p15IN□4b and p21 (23,24,47), and they were identified among differentially expressed and regulated genes in LSMC and MSMC by TGF-β (21,22). With respect to Gas1, to the present inventors' knowledge, this observation is the first to demonstrate Gas1 expression in human uterine tissue and its regulation by TGF-β. GAS1 acts as a negative regulator of the cell cycle and has been positively correlated with the inhibition of endothelial cell apoptosis and the integrity of resting endothelium (64). S imilar to p15, p21 and p27, myc suppresses the expression of GAS1 by limiting myc-max heterodimers binding to their promoters, (65,66). GAS1 is also reported to suppress growth and tumorigenicity of human tumor cells, and overexpression of MDM2, or p53 mutation inhibits Gas1-mediated action (68). The present inventors have identified max and MDM2 expression in LSMC and MSMC and their regulation by TGF-β, suggesting their potential interactions in leiomyoma cellular environment. It was previously reported that TGF-β isoforms stimulate DNA synthesis, but not cell division in LSMC and MSMC, suggesting that p27, p57 and Gas1, as well as the products of other cell cycle regulators, may influence the effect of TGF-β action on leiomyoma cell growth late in the S to M phases of the cell cycle transition. Collectively, the identification of several genes in this category, whose products regulate cell cycle progression as target of TGF-β autocrine/paracrine action in LSMC and MSMC, further indicate the importance of TGF-β in regulating the balance between cell proliferation, cell cycle arrest and apoptosis whose outcome directs leiomyoma growth and/or regression.

Expression and activation of various components of signal transduction pathways are essential for mediating the cellular actions of growth factors, cytokines, chemokines, polypeptide hormones, and adhesion molecules. The present inventors identified several genes functionally belonging to this category as differentially expressed and regulated in LSMC and MSMC in response to TGF-β action, among them are member of family of Ras/Rho, Smads and MAPK, guanine nucleotide binding protein alpha, GTP-binding protein overexpressed in skeletal muscle, PTK2 protein tyrosine kinase 2, S100 calcium-binding protein A5, adenylate cyclase 9, CDC-like kinase 2, Cdc42 effector protein 4, retinoic acid induced 3, receptor tyrosine kinase-like

orphan receptor 1, LIM protein and LIM domin kinase 2, phosphodiesterase 4D (cAMP-specific), protein phosphatase alpha, serine/threonine kinase 17a (apoptosis-inducing), focal adhesion kinase 2, STATs, *etc*. Although, Smad and MAPK pathways are known to be recruited and activated by TGF-β receptors, including in LSMC and MSMC, the components of other pathways are not the target of TGF-β. However, many growth factors, cytokines, chemokines, polypeptide hormones and adhesion molecules, expressed by LSMC and MSMC, either alone or through crosstalk with TGF-β receptor signaling may activate various components of the other pathways (1,20-22), although only the expression and activation of a few of these molecules has been demonstrated in leiomyoma and myometrium, and in LSMC and MSMC. Since GPRK5 expression was detected in leiomyoma and myometrium and was the target of GnRHa action in LSMC and MSMC (22), the present inventors further investigated and found GPRK5 expression is regulated by TGF-$\tilde{\beta}$. The biological implication of GPRK5 and regulation by TGF-β in LSMC and MSMC is unclear; however, GPKs serve as negative regulators of GPCR mediated biological responses through the generation of second messengers, such as cAMP and calcium/calmodulin, and down-regulation of their activity (desensitization)(69-71). Activation of calcium/calmodulin is reported to alter Smad function, with inhibition of calmodulin resulting in an increase in activin-dependent induction of target genes, whereas its overexpression decreased activin- and TGF-βaction (23, 24, 47). The result suggests that GPRK may act as downstream regulator of TGF-β receptor singling possibly through modulation of PKC, MAPK and/or calmodulin and hence influencing TGF-β autocrine/paracrine action in leiomyoma.

Tissue remodeling is also a critical step in progression of fibrotic disorders and modulation of ECM, adhesion molecules and protease expression, and phenotypic changes toward a myofibroblastic phenotype are essential components of this process (1,72-75). In this study and the previous study, the presenti inventors identified the expression of several genes in this category in leiomyoma and myometrium, as well as LSMC and MSMC including fibronectin, collagens, decorin, versican, desmin, vimentin, fibromodulin, several member of intergrin family, desmoplakin, extracellular matrix protein 1, enhancer of filamentation 1, porin, SPARC-like 1, syndecan 4, endothelial cell-specific molecule 1, as well as MMPs, TIMPs and ADAMs (21,22). The expression of fibronectin, vimentin, collagen type 1, fibromodulin,

MMP1, MMP2 and MMP9, TIMPs in leiomyoma and myometrium has been demonstrated and showed that TGF-β, through the activation of MAPK, regulates the expression of some of these genes (11,46,76). Of particular interest are the elevated expression of decorin, vimentin and fibromodulin in leiomyoma since they are considered to regulate the outcome of tissue fibrosis and their ability to bind TGF-β, thus controlling TGF-β autocrine/paracrine action (1,20,77,78). Since leiomyoma is believed to derive from transformation of myometrial connective tissue fibroblast and/or smooth muscle cells, the expression of vimentin in leiomyoma/LSMC imply that these cells have adopted a myofibroblastic characteristic. While granulation tissue myofibroblasts are derived from local fibroblasts, other cell types including smooth muscle cells have the potential to acquire a myofibroblastic phenotype (35,72-74). These cells express various cytokines including GM-CSF, IL-11 and TGF-β of which GM-CSF is considered to participate in fibroblasts transformation into myofibroblasts and enhancing their TGF-β expression (72-74). It has been shown that GM-CSF is a key regulator of TGF-β in LSMC, and their interaction and as well as the involvement of other cytokines such as IL-11 and IL-13 regulate various events leading to leiomyoma formation and progression of fibrosis (11,19). IL-11 either alone or through the induction of TGF-β is reported to alter myofibroblasts ECM turnover resulting in the progression of tissue fibrosis (35,79). Despite the importance of tissue turnover in the pathophysiology of leiomyoma, little data are currently available of the extent of ECM expression and the difference that may exist compared to myometrium, that contribute to the fibrotic characteristic of leiomyoma.

In conclusion, as a continuation of work with TGF-β, the present inventors have provided the first large-scale example of gene expression profile of LSMC and MSMC identifying specific cluster of genes whose expression is targeted by autocrine/paracrine action of TGF-β. The present inventors validated the expression of a selective number of these genes functionally recognized to regulate inflammatory response, angiogenesis, cell cycle, apoptotic and non-apoptotic cell death, and ECM matrix turnover, events that are central to leiomyoma pathobiology. Based on the present work and previous work with TGF-β, the present inventors propose that the individual and combined action of TGF-$\beta$with other profibrotic cytokines such as IL-11, orchestrate local inflammatory responses mediated through and influenced by the

expression of genes whose products regulate cell cycle progression, angiogenesis, apoptosis and tissue turnover, providing an environment leading to the progression of fibrosis.

Example 8—Differential Expression of Fibromodulin and Abl-interactor 2 in Leiomyoma and Myometrium and Regulation by Gonadotropin Releasing Hormone Analogue (GnRHa) Therapy

To validate the expression of fibromodulin and Abl-interactor 2 (Abi-2) that were identified as being differentially expressed in leiomyomata and myometrium and were regulated by GnRHa therapy. Fibromodulin is considered to have an anti-fibrotic role in wound repair and may be a biologically relevant modulator of TGF-beta activity during scar formation. Abl-interactor 2 encodes a non-receptor tyrosine kinase, c-Abl, that has been implicated in a variety of cellular processes including cell growth, reorganization of cytoskeleton, cell death and stress responses. Accordingly, a prospective study determining the tissue gene expression profile of myometrium and elimyoma using Real-time polymerase chain reaction (PCR) was carried out. Portions of leiomyoma and matched unaffected myometrium were collected from premenopausal women (N=27) who were scheduled to undergo hysterectomy for indications related to symptomatic leiomyoma. Seven of the patients received GnRHa therapy for three months prior to surgery. The untreated patients did not receive any medications (including hormonal therapy) during the 3 months prior to surgery.

Based on endometrial histology and the patient's last menstrual period, the tissue samples were identified as being from the proliferative (N=8) or the secretory (N=12) phase of the menstrual cycle. Total RNA was isolated and subjected to Real-time PCR. The results were analyzed using unpaired Student-test and Tuckey test (ANOVA) with a probability level of $P<0.05$ considered significant. Results are shown in Figures 20 and 21. These results for the first time document expression of fibromodulin and Abi-2 in leiomyoma and myometrium and provide evidence that the expression of these genes is influenced by ovarian steroids and possibly by a direct action of GnRHa on myometrial and leiomyoma cells.

All patents, patent applications, provisional applications, and publications referred to or cited herein, whether supra or infra, are incorporated by reference in their entirety, including all figures, tables, and sequences, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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Table 1

Gene Accession #	Gene Symbol	Change in Expression LYM vs MYM (P ≤0.02)
Transcription factors		
AB020634	NFAT5	+
M97388	DR1	+
U26914	RREB1	+
AF040253	SUPT5H	-
AB002386	EZH1	-
L38933	HUMGT198A	-
AB022785	ASH2L	-
AB014558	CRY2	-
Cell cycle regulators		
X60188	MAPK3	-
U66469	CGRRF1	-
Cell adhesion receptors/proteins		
AF106861	ATRN	+
Z29083	TPBG	+
AB002382	CTNND1	-
Extracellular transport/carrier proteins		
U09210	SLC18A3	+
Oncogenes and tumor suppressors		
X57110	CBL	+
M16038	LYN	+
X60287	MAX	+
U96078	HYAL1	-
Stress response proteins		
W28616	HSPCB	+
X83573	ARSE	-
D87953	NDRG1	-
Membrane channels and transporters		
AF027153	SLC5A3	+
M55531	SLC2A5	+
X57303	SLC7A1	+
X91906	CLCN5	-
Extracellular matrix proteins		
U05291	FMOD	-
AB011792	ECM2	-
Trafficking/targeting proteins		
D89618	KPNA3	+
AC004472	VCP	+
AA890010	SEC22L1	+
L43964	PSEN2	+
X97074	AP2S1	+
AA192359	TNPO3	+
U32315	STX3A	-
Metabolism		
D50840	UGCG	+
M21186	CYBA	+
AC005329	NDUFS7	+
U44111	HNMT	+
M84443	GALK2	+
X14608	PCCA	+
AF014402	PPAP2A	+
AF035555	HADH2	+
U84371	AK2	+
AA526497	UQCRH	+
AI557064	NDUFV2	+
D55654	MDH1	+
AL049954	AHCYL1	-
AA420624	MAOA	-
M93107	BDH	-
Post-translational modification		
U84404	UBE3A	-
Translation		
L36055	EIF4EBP1	+
Apoptosis associated proteins		
Z70519	TNFRSF6	+
AJ006288	BCL10	+
U04806	FLT3LG	-
RNA processing, turnover, and transport		
U40763	PPIG	+
AB007510	PRPF8	-
X85237	SF3A1	-
U76421	ADARB1	-
Cell receptors (by ligand)		
J03171	IFNAR1	+
M33210	NDRG1	-
AJ225028	GABBR1	-
D15050	TCF8	-
AF030339	PLXNC1	-
Intracellular transducers/modulators		
AB007881	SMG1	+
AB004904	SOCS3	+
D89094	PDE5A	+
Z50053	GUCY1A2	+
X95632	ABI2	+
Y13493	DYRK2	+
D88532	PIK3R3	+
Y18206	PPP1R3D	+
M96995	GRB2	+
AF015254	AURKB	+
U02680	PTK9	+
AF052135	STAMBP	+
U46461	DVL1	+
AB003698	CDC7	+
AI961669	ARFGEF2	+
X70218	PPP4C	+
X99325	STK25	+
L36151	PIK4CA	-
AL049970	PRKRIR	-
AI671547	RAB9A	-
AF103905	RAPGEF3	-
X95735	ZYX	-
M33552	LSP1	-
X62048	WEE1	-
S76965	PKIA	-
U25771	ARF4L	-
AF035299	DOK1	+
Protein turnover		
X87212	CTSC	+
AL080090	ANAPC10	+
AJ132583	NPEPPS	-
AF099149	ARIH2	-
Cell receptors (by activities)		
AF084645	NR1I2	+
AB020639	ESRRG	+
Cytoskeleton/motility proteins		
AB008515	NOL7	+
AI056696	CETN3	-
Functionally unclassified		
AF035444	PHLDA2	+
U79299	OLFM1	+
U22963	MR1	+
U15552	HSU15552	+
AB015332	AKAP8L	+
AF068195	UBADC1	+
AB011542	EGFL5	+
Z78368	C1orf8	-
AF053356	LRCH4	-
AF009426	C18orf1	-
not classified		
AB011096	SARM1	+
AJ236885	ZNF148	+
N42007	NUP50	+
Z48570	DDX24	+
M19650	CNP	+
AB002348	KIAA0350	+
AB014564	KIAA0664	-
M29551	PPP3CB	-
AB020699	KIAA0892	-
AB002370	KIAA0372	-
AB023181	DLGAP4	-
AB011106	ATRNL1	-
D88152	SLC33A1	-
AF082657	ERAL1	+
AB023163	HIP14	-
AF040964	C4orf15	+
U33838	RELA	+
M22919	MYL6	-
U93869	POLR3F	+
X59417	PSMA6	+
AJ224326	RPE	+
U60644	PLD3	+
AB018257	ZNF294	-

Table 2

Gene Accession#	Gene Symbol	Change in Expression LYM vs MYM (P ≤0.02)
Cell surface/Matrix Protein		
D26579	ADAM8	+
Transcription Factor		
U15655	ERF	+
L39059	TAF1C	+
M96577	E2F1	+
AF025654	RNGTT	-
U15642	E2F5	-
AB015132	KLF7	-
U63810	CIAO1	-
U52960	SURB7	-
U65093	CITED2	-
AJ001183	SOX10	-
Cell cycle		
U03106	CDK1A	+
L23959	TFDP1	-
M80629	CDC2L5	-
X77794	CCNG1	-
Cell adhesion receptors/proteins		
AF007194	Mucin 3	+
X15606	ICAM2	-
D14705	CTNNA1	-
S66213	ITGA6	-
Oncogenes and tumor suppressors		
U96078	HYAL1	-
Stress response proteins		
AI972631	ARS2	-
Membrane channels and transporters		
X89066	TRPC1	-
AB021981	SLC35A3	-
D50312	KCNJ8	-
Extracellular matrix proteins		
U37283	MFAP5	-
Trafficking/targeting proteins		
AF002163	AP3D1	+
X96783	SYT5	-
Metabolism		
AJ004832	NTE	+
AF062529	NUDT3	+
D38537	PPOX	+
AI345944	NDUFB1	-
AI766078	COQ7	-
D14710	ATP5A1	-
Post-translational modification		
U31525	GYG	-
Apoptosis associated proteins		
Y09392	TNFRSF25	+
AF015451	CFLAR	-
M16441	LTA	-
RNA processing, turnover, and transport		
L35013	SF3B4	+
AJ007509	HNRPUL1	+
AF016369	PRPF4	-
M96954	TIA1	-
Chromatin proteins		
AF045184	SKIIP	-
Cell Surface receptors		
X06614	RARA	+
AF109134	OGFR	+
D16827	SSTR5	-
X61615	LIFR	-
M64347	FGFR3	-
M15169	ADRB2	-
U23850	ITPR1	-

Gene Accession#	Gene Symbol	Change in Expressionn LYM vs MYM (P ≤0.02)
Growth Factor/Cyt/Chemo/Polypept-Horm		
U79716	RELN	+
M63582	TRH	+
M13982	IL4	+
X52599	NGFB	+
Intracellular transducers/modulators		
U39064	MAPKK6	+
X82260	RANGAP1	+
Z15108	PRKCZ	+
R54564	MINK	+
U09284	LIMS1	+
U12779	MAPKAPK2	+
U18420	RAB5C	+
AL050268	RAB1A	-
AB005047	SH3BP5	-
X52213	LTK	-
	GNEF1	-
D85758	ERH	-
AF014398	IMPA2	-
AJ011736	GRAP2	-
U59913	SMAD5	-
X17576	NCK1	-
U48730	STAT5B	-
U17743	MAP2K4	-
U43885	GAB1	-
Protein turnover		
D49742	HABP2	+
U80034	MIPEP	-
Cytoskeleton/motility proteins		
W27148	MAP1B	-
DNA synthesis, recombination, repair		
X91992	ALKBH	-
Y15572	RAD51L3	-
AF007871	DYT1	-
AF058696	NBS1	-
Functionally unclassified		
AI924594	TSPAN-2	-
Z68747	mitochondrial ribosomal protein S31	-
AB018285	zinc finger protein	-
Not classified		
D42085	NUP93	-
D87437	C1orf16	+
X77548	NCOA4	-
D79990	RASSF2	-
U05861	AKR1C1	-
L49054	MLF1	-
AB007884	ARHGEF9	-
AF044896	C1orf38	-
AJ223352	HIST1H2BK	-
AA043348	HSPA4	-
Z85986	C6orf69	-
W26677	FLJ35827	+
AB011133	MAST3	+
AB018274	LARP	+
U92896	EFNA2	+
AF064801	RNF139	+
U47924	GRCA	-
AB007896	KIAA0436	-
AJ002428	VDAC1	-

Table 3

Gene Accession #	Gene Symbol	Change in Expression ($p \le 0.02$)
Cell surface antigens		
X84746	ABO	+
AF004876	YIF1	+
Transcription/activators/repressors		
X98253	ZNF183	-
D38251	POLR2E	-
U22431	HIF1A	-
AB002332	CLOCK	+
U33838	RELA	-
U15306	NFX1	-
AF040253	SUPT5H	+
L19067	RELA	+
M74099	CUTL1	+
U48436	FMR2	+
AA478904	KLF7	+
M69043	NFKBIA	-
Cell cycle-regulating kinases		
U17743	MAP2K4	-
D88357	CDC2	-
L04658	CDK5	-
X66357	CDK3	+
M74091	CCNC	-
L23959	TFDP1	-
Cell adhesion receptors/proteins		
X69819	ICAM3	-
Z29083	TPBG	-
AF007194	Mucin 3, Intestinal	+
Oncogenes and tumor suppressors		
J03069	MYCL2	+
X72631	NR1D1	+
U09577	HYAL2	-
AI743606	RAB8A	-
U04313	SERPINB5	+
AF013168	TSC1	+
Trafficking/targeting proteins		
X99459	AP3S2	-
AW044624	RER1	-
U60644	PLD3	-
AA890010	SEC22L1	-
AC004472	VCP	-
AF034546	SNX3	-
Z12830	SSR1	-
AF044671	GABARAP	-
Metabolism		
AC005329	NDUFS7	-
M22976	CYB5	+
AF047181	NDUFB5	-
D16294	ACAA2	-
AI345944	NDUFB1	-
D14710	ATP5A1	-
X06994	CYC1	-
AI540957	QP-C	-
AI557064	NDUFV2	-
U19822	ACACA	+
AF047469	ASNA1	-
Protein modification enzymes		
D29643	DDOST	-
AD000092	CALR	-
AF035280	EIF2B2	-
L36055	EIF4EBP1	-
L34600	MTIF2	-
D28483	RBMS2	-
RNA processing/ turnover/ transport		
U51334	TAF15	+
D59253	NP25	-
Z48501	PABPC1	-
L36529	THOC1	+
AF083190	DNAJC8	+
D28423	SFRS3	-

Gene Accession #	Gene Symbol	Change in Expression ($p \le 0.02$)
Growth factors/cytokines/ chemokines		
J00219	IFNG	+
U32324	IL11RA	+
Z70519	TNFRSF6	-
X04571	EGF	+
X72308	CCL7	+
X78686	CXCL5	+
J04513	FGF2	-
S74221	IK	-
U43368	VEGFB	+
AL021155	NPPA	+
Intracellular transducers/modulators		
X75958	NTRK2	+
S76475	NTRK3	+
U43885	GAB1	-
X84709	FADD	-
M96995	GRB2	-
U46461	DVL1	-
AF051323	SCAP2	-
X66363	PCTK1	-
AB018330	CAMKK2	+
L13616	PTK2	-
U02680	PTK9	-
X72964	CETN2	-
Y17711	CBARA1	-
U51004	HINT1	-
U94747	HAN11	-
U78733	SMAD2	-
GTP/GDP/G-protein/GTPase modulators		
D13988	GDI2	-
U18420	RAB5C	+
U34806	GPR15	+
U18550	GPR3	-
Amino- and carboxypeptidases		
L13977	PRCP	-
Metalloproteinases		
U80034	MIPEP	-
Proteasomal proteins		
D26600	PSMB4	-
AB009398	PSMD13	-
X59417	PSMA6	-
D26598	PSMB3	-
D38048	PSMB7	-
Cytoskeleton/motility proteins		
AB007862	PCNT2	+
U48734	ACTN4	+
U01828	MAP2	+
U39226	MYO7A	+
AI540958	DNCL1	+
AF020267	MYO9B	+
U43959	ADD2	+
AL096717	EML2	+
AI961040	TUBGCP2	+
Extracellular matrix and carrier proteins		
M12625	LCAT	+
AF093118	FBLN5	+
M20776	COL6A1	-
U80034	MIPEP	-
AB006190	AQP7	+
AB021981	SLC35A3	-
U90313	GSTO1	-
X67301	IGHM	-
M92303	CACNB1	+
X91906	CLCN5	+
AB023173	ATP11B	+
M20471	CLTA	-
U27467	BCL2A1	+
U30872	CENPF	-
AI857458	UCN	-

Gene Accession #	Gene symbol	Change in Expression ($p \le 0.02$)
D87432	SLC7A6	+
N80906	CST6	+
D38535	ITIH4	+
M31767	MGMT	+
AB007884	ARHGEF9	-
AC004472	KIAA1539	-
Functionally unclassified		
W28869	TEGT	-
Z68747	MRPS31	-
L07758	PWP1	-
AJ007014	NCBP2	-
U72508	B7	-
AA524058	C6orf74	-
D86062	C21orf33	-
D87343	DSCR3	+
AF042384	BC-2	-
AF068195	UBADC1	-
AL021937	RFPL3S	+
U80744	TNRC5	-
AF035444	PHLDA2	-
not classified		
AL031177	APG4A	+
AB007884	ARHGEF9	-
AC004472	KIAA1539	+
AF040964	C4orf15	-
D87742	FLJ39207	+
AB006628	FCHO1	+
AB014592	KIAA0692	+
AB023214	ZBTB1	+
AB028964	FOXJ3	+
U54999	GPSM2	+
L49054	MLF1	-
AA926959	CKS1B	+
Ras-Like Protein Tc4		-
AB002292	ARHGEF10	+
M24899	THRA	+
U92896	EFNA2	+
AJ222967	CTNS	+
AL031983	OR2H3	+
U05681	BCL3	+
AF014398	IMPA2	-
X67325	IFI27	-
U90907	PIK3R3	-
AF030107	RGS13	+
AL049634	PTPNS1L2	+
AF091071	RER1	+
AC005525	IGSF4C	+
U49278	UBE2V1	-
U39318	UBE2D3	+
AF075599	UBE2M	-
AJ002428	VDAC1	-
U84388	CRADD	-
X63657	FVT1	+

Table 4

Gene Accession #	Gene Symbol	Change in Expression ($p \leq 0.02$)
Cell surface/Matrix protein		
AF106861	ATRN	-
AJ001683	KLRC4	-
D26579	ADAM8	-
M33308	VCL	+
U12255	FCGRT	+
Transcription Factors		
AJ001183	SOX10	-
AB004066	BHLHB2	-
AF012108	NCOA3	-
AF025654	RNGTT	-
AF035262	SMARCE1	-
D42123	CRIP2	-
D80003	NCOA6	-
L19067	RELA	+
L19871	ATF3	+
L38933	HUMGT198A	+
L39059	TAF1C	+
L49380	SF1	+
M81601	TCEA1	+
U37251	ZNF177	+
U63810	CIAO1	+
U68727	PKNOX1	+
X99720	PRCC	+
Metabolism		
AF104421	UROD	-
AL049954	AHCYL1	-
D16294	ACAA2	-
D16481	HADHB	-
D28137	BST2	-
D38537	PPOX	-
D55639	KYNU	-
U25849	ACP1	+
U91316	BACH	+
X58965	NME2	+
X76228	ATP6V1E1	+
RNA processing transport		
AA205857	SNRPD3	-
AB007510	PRPF8	-
AB017019	HNRPDL	-
AL008726	ZSWIM3	-
U40763	PPIG	+
Growth factor/chemokine and receptors		
X78686	CXCL5	-
X81882	CUL5	-
D13168	EDNRB	-
D14582	EPIM	-
D26070	ITPR1	-
J03278	PDGFRB	-
J03634	INHBA	-
M91211	AGER	+
S67368	GABRB2	+
U23850	ITPR1	+
U78110	NRTN	+
X06614	RARA	+
X60592	TNFRSF5	+
X64116	PVR	+
Non-receptor protein kinases		
AI341656	LIM	-
L13738	ACK1	+
L27071	TXK	+
X54637	TYK2	+
Non-receptor phosphatases		
AI739548		-
J03805	PPP2CB	-
L36151	PIK4CA	+
M29893	RALA	+
M64929	PPP2R2A	+
X68277	DUSP1	+

Gene Accession #	Gene Symbol	Change in Expression ($p \leq 0.02$)
Nuclear receptors		
AB020639	ESRRG	-
AF084645	NR1I2	-
AF109134	OGFR	-
X75918	NR4A2	+
Translation/post-trans modification		
D84273	NARS	-
M34539	FKBP1A	+
Death receptor proteins/adaptors		
AF006041	DAXX	-
U04806	FLT3LG	+
U50062	RIPK1	+
X98176	CASP8	+
Chaperones/ heat shock proteins		
W28616	HSPCB	-
L26336	HSPA2	+
X04106	CAPNS1	+
Cell signaling/EC communication		
AI658639	ENSA	-
L19605	ANXA11	+
M32886	SRI	+
U37283	MFAP5	+
U79716	RELN	+
Adaptor/receptor-associated proteins		
AF015767	BRE	-
U09284	LIMS1	+
GTP/GDP and G-protein GTPase activity modulators		
AB002349	RALGPS1	-
AI961929	ARHGAP1	-
M85169	PSCD1	+
U57629	RPGR	+
Trafficking/targeting proteins		
AF002163	AP3D1	-
D63476	ARHGEF7	-
U00957	AKAP10	+
X07315	NUTF2	+
DNA replications		
J05249	RPA2	+
L20046	ERCC5	+
L26336	HSPA2	+
L26339	RCD-8	+
L78833	VAT1	+
M62302	GDF1	+
M84820	RXRB	+
Other functional protein		
M20681	SLC2A3	-
AA631972	NK4	-
AB026891	SLC7A11	-
AF047472	BUB3	-
AI972631	ARS2	-
AL008726		-
AL050254	FBXO7	-
D44466	PSMD1	-
D87953	NDRG1	-
L43964	PSEN2	+
M76558	CACNA1D	+
M83664	HLA-DPB1	+
M95178	ACTN1	+
U40705	TERF1	+
U59913	SMAD5	+
U72263	EXT2	+
X01703	TUBA3	+
X14487	KRT10	+
X51602	FLT1	+
X58199	ADD2	+
X76538	MPV17	+
X78338	ABCC1	+
Z24727	TPM1	+

Gene Accession #	Gene Symbol	Change in Expression ($p \leq 0.02$)
Functionally unclassified		
AA923149	WSB2	-
AB002322	SRRM2	-
AB007879	CP110	-
AB007890	LKAP	-
AB007915	KIAA0446	-
AB007931	RBAF600	-
AB011133	MAST3	-
AB011151	BDG29	-
AB014515	N4BP1	-
AB014564	KIAA0664	-
AB014599	BICD2	-
AB018344	DDX46	-
AB023186	PEPP3	-
AB028995	PPM1E	-
AB028998	TENC1	-
AB029012	EST1B	-
AF051941	NME6	-
AF058696	NBS1	
AL031228	VPS52	-
AL031282	FLJ13052	-
AL046940	FLJ46603	-
D29677	HELZ	-
D50645	SDF2	-
D50920	THRAP4	-
D79990	RASSF2	-
D87119	TRIB2	-
Not classified		-
S59184	*RYK*	+
U01062	*ITPR3*	+
U12597	*TRAF2*	+
U41737		+
U85611	*CIB1*	+
U89358	*L3MBTL*	+
U93869	*POLR3F*	+
W25974	*MTX1*	+
W27949	*HEBP2*	+
X16281	*ZNF44*	+
X52851	*PPIA*	+
X65784	*SPG7*	+
X92814	*HRASLS3*	+
XM29054		+
Y09305	*DYRK4*	+
	GEF	+
Protein Kinase Pitslre, Alpha,		
Proto-Oncogene N-Cym		+
Single-Stranded DNA-Binding		+
Protein Mssp-		+

Table 5

Gene Symbol	Gene Name	Ref#9	Ref#11	Ref#12	Ref#14
BCL10	B-cell CLL/lymphoma 10	-	+	-	-
CDH2	Cadherin 2A	+	-	-	-
F13A1	Coagulation factor XIII	-	-	+	-
CRH	Corticotropin Releasing Hormone	-	+	-	-
ECM2	Extracellular Matrix Protein 2	+	-	-	-
HOXD4	Homeo box D4	-	-	-	+
ENO1	c-myc binding protein	-	-	-	+
PIPPIN	Ortholog of rat Pippin	-	-	-	+
PPIB	Peptidylprolyl isomerase B	-	-	-	+
RY1	Putative ucleic acid binding protein	-	-	-	+
TYMS	Thymidylate synthetase	+	+	-	+

Table 6

Gene Accesssion #	Gene Symbol	Gene Accesssion #	Gene Symbol
	Transcription activators/repressors		*Intracellular kinases (non-receptor)*
AJ000041	HOXC11	AF-068864	PAK3
NM_001130	AES	L13616	PTK2
NM_006164	NFE2L2	NM_003177	SYK
	Cell cycle-regulating kinases	NM_002822	PTK9
M84489	MAPK1	NM_012290	TLK1
	Oncogene/tumor suppressors		*GPs/GTPase activity modulators*
NM_002315	LMO1	M28212	RAB6A
M24898	NR1D1	AF030107	RGS13
NM_002350	LYN		*Kinase activators/inhibitors*
	Membrane channels and transporters	X82240	TCL1A
NM_006358	SLC25A17	NM_003629	PIK3R3
	Trafficking		*Cytoskeleton/motility proteins*
NM_005829	AP3S2	X58199	ADD2
	Metabolism		*Functionally unclassified*
NM_001355	DDT	NM_004487	GOLGB1
NM_000819	GART	NM_004337	C8orf1
NM_004317	ASNA1	NM_006992	B7
	Translation/post-translational		*Not classified*
NM_006156	NEDD8	NM_021964	ZNF148
NM_003758	EIF3S1	NM_021999	ITM2B
	Death receptor-associated proteins	NM_014629	ARHGEF10
AF015956	DAXX	NM_030913	SEMA6C
	RNA processing/turnovert	NM_012263	TTLL1
NM_002568	PABPC1	NM_020150	SARA1
	Neuropeptides/growth factors		PP1A
NM_003353	UCN		RPE
NM_002006	FGF2		MAFK
	Extracellular communication		LRIG2
NM_001405	EFNA2		DKFZP586F242
NM_004279	EEEF1E1		KIAA0290
	Intracellular transducers/effectors		Homeotic Protein Hox5.4
NM_005079	TPD52		
NM_006012	CLPP		

Table 7

Gene Accession# Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accession# Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001	Gene Accession# Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001
BC003576 ACTN1	-	AF007132 ABHD5	-	AC006020 AASS	+
Adenylyl Cyclase-AP2	+	AL831821 ACADSB	+	AF245699 AGTR1	+
M12271 ADH1A	+	AJ306929 AFURS1	-	AC002366 AMELX	+
AB014605 AIP1	+	AB031083 AKR1C1	+	D12775 AMPD3	+
BC000171 AMD1	-	AC002366 AMELX	+	AB084454 ANGPT1	+
AK092006 ANXA2	+	AB084454 ANGPT1	+	AF019225 APOL1	+
BC001429 ANXA5	-	AF168956 APLP2	-	BC014450 B7	+
AK098588 APEX1	+	AF047432 ARF6	+	AB004066 BHLHB2	-
AF038954 ATP6V1G1	-	AK000379 ASNS	-	AB062484 CALD1	+
AB020680 BAG5	-	AF022224 BAG1	+	AB023172 CARD8	+
AF019413 BF	+	BC019307 BCL2L1	+	BC002609 CBX1	-
AB004066 BHLHB2	-	AC006378 BET1	+	AF213700 CDKN1B	+
BC009050 BTG1	+	AB004066 BHLHB2	-	AF018081 COL18A1	+
AB030905 CBX3	+	AF002697 BNIP3	-	BC000326 COPB2	+
BC008816 CCBP2	+	AL021917 BTN3A3	+	AF062536 CUL1	-
BC032518 CCNG2	+	AB059429 BUCS1	+	NM_005491 CXorf6	-
AU130185 CDH6	+	AJ420534 C6orf145	-	AC004634 DTR	-
AJ011497 CLDN7	-	AF111344 CASP10	+	AA053720 EDIL3	+
AJ006267 CLPX	+	AK022697 CBARA1	-	AF174496 EEF1A1	+
BC005159 COL6A1	-	BC009356 CDC42EP1	-	AF139463 EGR2	-
AK098615 CRY1	-	AF002713 CENPB	-	N66802 EGR3	-
AL833597 CSF2RA	-	AK128741 CHD4	+	AF000670 ELF4	-
AF013611 CTSW	-	AF136185 COL17A1	+	AF083633 EXTL1	-
AK025446 DKFZP564M182	-	AB014764 COPS7A	-	BC001786 FKBP4	-
AJ005821 DMXL1	-	AF452623 CRELD1	-	AY358917 FSTL3	-
AF088046 DNAJA2	-	AK098615 CRY1	-	AB014560 G3BP2	-
BC039596 DNM2	-	AL833597 CSF2RA	-	AK022142 GAB1	+
AF139463 EGR2	-	AB014595 CUL4B	+	AF169253 GATA2	-
N66802 EGR3	-	AB015051 DAXX	-	AL031659 GHRH	+
AF001434 EHD1	-	AJ313463 DF	+	BC026329 GJA1	+
AF208852 EIF4A2	+	BC015800 DXYS155E	+	AF052693 GJB5	+
BC000738 EMD	-	BC014410 EFEMP1	-	AF493902 GNA13	+
AF103905 EPAC	+	AF139463 EGR2	-	K03460 H2-ALPHA	-
AF052181 EPIM	+	BC028412 ELL2	+	AF264785 HES1	-
BC003384 FKBP2	+	AK092872 ERCC2	+	AB017018 HNRPDL	+
AF085357 FLOT1	+	AK000818 FLJ20811	+	AF056979 IFNGR1	-
AY358917 FSTL3	-	AK074486 FLJ90005	-	AC005369 IK	+
L13698 GAS1	+	AK130009 FRZB	+	AJ271736 IL9R	+
AF169253 GATA2	-	AJ251501 GAD2	+	AF007140 ILF3	+
AF144713 GDI2	+	AC004976 GARS	-	AY351902 IQGAP2	+
AC000051 GGT1	+	AK094782 GLUD1	-	AB007893 KIAA0433	+
NM_000855 GUCY1A2	+	AF070597 GNB1	-	AB014528 KIAA0628	+
X83412 HAB1	+	AK023082 GORASP2	+	AB028956 KIAA1033	-
AF103884 HB-1	+	AF077204 GTPBP1	+	AB014581 L3MBTL	+
AF264785 HES1	-	BC035837 HAS1	+	BC016618 LCP2	+
BC022283 HFL3	+	AK097824 HSPA2	+	AF211969 LENG4	-
IGF1	+	BC009696 IFITM2	+	AF004230 LILRB1	+
D86989 IGL2	+	AC005369 IK	+	BC017263 LMAN2	+
AF038953 ITM2A	-	L25851 ITGAE	+	AF055581 LNK	-
NM_005354 JUND	-	AF003521 JAG2	-	AK095843 LOC169834	+
AB014765 JWA	+	BC002646 JUN	-	AB025247 MAFF	-
AB002308 KIAA0310	-	AF081484 K-ALPHA-1	-	AC005943 MBD3	-
AB014548 KIAA0648	+	AF056022 KATNA1	+	BC012396 MGC40157	+
AK129875 LAPTM4A	+	AK025504 KIAA0251	-	AF508978 MTA1	-
AB017498 LRP5	+	AB002301 KIAA0303	-	AK130664 MTHFD2	-
AF027964 MADH2	+	AB014528 KIAA0628	+	NM_005593 MYF5	-
AK026690 MADH3	+	AB014548 KIAA0648	+	AB020673 MYH11	+
AB025247 MAFF	-	AB040969 KIAA1536	-	BC005318 MYL1	+
AB025186 MAPRE3	-	AB040972 KIAA1539	-	AB014887 ORM1	+
AB017335 MAZ	-	AF061809 KRT16	+	AK125499 P5	+
AF061261 MBNL2	+	BC009971 KRTHA3B	+	AJ238420 PDGFA	-
BC012396 MGC40157	+	AB014581 L3MBTL	+	AK055119 PDK2	-
AF125532 MKNK2	+	AF000177 LSM1	+	AB051763 POR	-
BC001122 MSH2	+	AB025186 MAPRE3	-	AF042385 PPIE	+
AF508978 MTA1	-	AB018266 MATR3	-	AF345987 PRKCG	+
AF057354 MTMR1	+	AC005943 MBD3	-	M95929 PRRX1	-
NM_005593 MYF5	-	AY032603 MCM3	-	AF119836 RAB6A	+
AB011179 NCDN	-	AF508978 MTA1	-	AF019413 RDBP	+
AF047181 NDUFB5	+	AK130664 MTHFD2	-	AF055026 RPIP8	+
AB014887 ORM1	+	AB023192 NISCH	+	BC020740 SGCD	+
BC009610 PC4	+	AC004663 NOTCH3	-	AF519179 SMOX	-
AK023529 PCBP2	-	AB005060 NRG2	+	AF391283 SSA1	-
AB029821 PEMT	-	AK025458 NUCB1	-	BC012088 TAF10	-
AF254253 PHKG1	+	NCOR 2	-	BC000125 TGFB1	-
AF220656 PHLDA1	-	AF109134 OGFR	+	AF050110 TIEG	-
AF025439 PKM2	-	AJ238420 PDGFA	-	AY065346 TNFAIP1	-
A18757 PLAUR	-	AB005754 POLS	-	AF019413 TNXB	-
AB006746 PLSCR1	-	AB051763 POR	-	AK025459 TRA1	+
A24059 PNLIP	+	AA846273 PRCC	+	AJ440721 TXNDC5	+
AB005754 POLS	-	AF044206 PTGS2	-	AB062290 TYMS	+
AF042385 PPIE	+	AY449732 PTHR1	+	BC000379 UBB	+
BC047502 PPP1R3D	+	BC002438 RAB4A	+	AB003730 UBC	+
AK091875 PPP2CB	-	AF080561 RBM14	-	AF002224, UBE3A	-
AI800682 PTPN21	-	BC003608 RBPMS	-	AF001787 UCP3	+
BC028038 PTPRD	+	AL031228 RING1	+	AF135372 VAMP2	-
BC001390 QP-C	+	AB078417 RIS1	+	AB029013 WHSC1	-
BC003608 RBPMS	-	AK096243 RPN2	+	AB023214 ZBTB1	-
AF019413 RDBP	+	D10570 RUNX1	-	AF060865 ZNF205	+

Table 7

Gene Accession# Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accession# Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001	Gene Accession# Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001
AF086557 RPL10A	+	BC002829 S100A2	+	AF055077 ZNF42	+
AB007147 RPS2	+	AB011096 SARM1	+		
BC011645 RRAD	-	BC020740 SGCD	-		
D10570 RUNX1	-	AC004000 SLC25A5	-		
AB028976 SAMD4	-	AY142112 SLC4A3	+		
AF070614 SCHIP1	-	AF053134 SNCB	+		
BC005927 SERPINE1	-	AB061546 SRP14	+		
AJ000051 SF1	-	AK125542 SRPX	+		
AK097315 SF3B4	-	AB015718 STK10	+		
BC004534 SFPQ	-	BC012085 STK38	+		
AL110214 SFRS6	-	AF064804 SUPT3H	+		
AB020410 SHH	+	BC000125 TGFB1	-		
AB001328 SLC15A1	+	AI290070 THBS1	+		
AF519179 SMOX	-	AY117678 TPT1	+		
AK096917 SREBF2	-	AF062174 TRIAD3	-		
AF261072 TCBAP0758	+	BC014243 TYK2	-		
BC003151 TCFL1	+	AB003730 UBC	+		
BC000125 TGFB1	-	AB014610 USP52	+		
AF050110 TIEG	-	BC030810 ZNF230	-		
AF087143 TOP2B	+	AJ245587 ZNF248	+		
AC002481 TUSC4	+	BI547129 ZW10	-		
AC002400 UBPH	+				
AF060538 VAMP1	+				
AF134726 VARS2	-				
BC000165 VDAC2	-				

Table 8

Gene Accesion# Gene Symbol		GnRHa 2h vs TGF-βRII antisense p ≤0.001	Gene Accesion# Gene Symbol		GnRHa 6h vs TGF-βRII antisense p ≤0.001	Gene Accesion# Gene Symbol		GnRHa 12h vs TGF-βRII antisense p ≤0.001
AK000002	ABCC10	+	BC015961	ADM	+	BC000292	ACTG1	+
AF129756	AIF1	+	AF129756	AIF1	+	AF023476	ADAM12	+
AA114994	ARGBP2	+	AY341427	AP2B1	+	AF001042	ADARB1	-
BC014450	B7	-	BC004537	ATP6V0C	-	AB018327	ADNP	+
AB005298	BAI2	-	BC008861	ATP6V0D1	-	AF245699	AGTR1	+
AF090947	BBS4	+	AB009598	B3GAT3	+	AF129756	AIF1	+
AB038670	BDNF	+	AB029331	C6orf18	+	D45915	ALK	+
AC006378	BET1	+	AF078803	CAMK2B	+	AK057883	AP2M1	+
AB018271	BPAG1	+	BC015799	CASP7	+	AK023088	ARL6IP	-
AC000391	BRD3	+	AB025105	CDH1	+	AF001307	ARNT	+
AF016270	BRD8	+	AB001090	CDH13	+	AB018271	BPAG1	+
AI420534	C6orf145	-	AB037187	CHST7	+	AK096489	BZW1	+
AB029331	C6orf18	+	AK122769	CKMT2	+	AB029331	C6orf18	+
AF072164	C9orf33	+	AB032372	CKTSF1B1	+	AF037335	CA12	+
AC002543	CAPZA2	-	AF000959	CLDN5	+	AF070589	CACNA1C	-
BC015799	CASP7	+	AF053318	CNOT8	+	BC005334	CETN2	+
BC036787	CTF1	-	BC022069	CRABP1	+	AY497547	CMKLR1	+
AF280107	CYP3A5	+	BC003015	DGCR14	+	NM_001886	CRYBA4	+
BC000485	DDC	-	BC038231	DUSP8	+	AF361370	DIA1	+
AB018284	EIF5B	+	BC020746	DXS1283E	+	AF498961	DRD1	+
AF253417	EPHX1	-	J03066	EN2	+	AK057845	EFNA1	+
AI879202	ETHE1	-	BC002706	ERBB3	-	AI879202	ETHE1	-
BC001325	FUBP3	-	BC002706	ERBB3	-	AC002389	GAPDS	+
AB058690	GPS2	+	AI879202	ETHE1	-	AF015257	GPR30	+
AY136740	GPSM2	+	AF241235	FXYD2	+	AF103803	H41	-
NM_000855	GUCY1A2	+	AF124491	GIT2	+	X83412	HAB1	+
X83412	HAB1	+		Glial Growth Factor 2	+	BC005240	HAX1	+
	HERV-K(HML6)	-	AL133324	GSS	+	AK058013	HPGD	+
AF299094	HSF1	-	AB032481	HOXD13	+	BC000290	IGHMBP2	+
AY136751	HTR2B	+	AF299094	HSF1	-	BC015752	IRF4	+
BC015335	ICT1	+	AF441399	HSGP25L2G	+	AK074047	ITGAX	+
AF011889	IDS	+	AF275719	HSPCB	+	AF135158	JIK	+
BC002793	IFNAR2	-	AB030304	HUMGT198A	+	AF233882	JUP	-
AF117108	IMP-3	+	BC014972	IL2RG	+	AB020638	KIAA0831	+
AF003837	JAG1	+	AB012853	ING1L	+	AF115510	LRRFIP1	-
AF072467	JRK	+	AF361886	KEAP1	-	AF010193	MADH7	-
AF361886	KEAP1	-	BC005407	KIAA0169	+	AL137667	MAPK8	+
AB014564	KIAA0664	-	BC014932	KIAA0280	-	AK025602	MGC2747	+
BC034041	LMO2	+	AB007887	KIAA0427	-	AF125532	MKNK2	+
AK074703	LOC89944	+	AB028953	KIAA1030	+	BC006491	MPZ	+
AF000177	LSM1	+	BC014781	LCAT	+	AB051340	MRPL23	+
AK025599	MAN1A1	+	AB016485	LOB1	-	AF113003	NCOR2	-
AK124738	MAP4K5	+	AF072814	M96	+	AF013160	NDUFS2	+
AK025602	MGC2747	+	AF010193	MADH7	-		E6-Ap.	-
AB037859	MKL1	-	AL137667	MAPK8	+	Papillomavirus		+
AF102544	MOCS3	-	AY032603	MCM3	-	BC011539	ORC1L	+
BC006491	MPZ	+	AL137295	MLLT10	+	BC000398	PAFAH1B2	+
AB037663	MYLK	+	AB051340	MRPL23	+	AL117618	PDHB	+
AF113003	NCOR2	-	AB046613	MYL6	+	AB002107	PER1	-
AF044958	NOUFB8	+	NM_004998	MYO1E	+	BC062602	PNN	-
BC002421	NEF3	+	AF113003	NCOR2	-	AK095191	POU6F1	-
AB010710	OLR1	-	AF013160	NDUFS2	+	BC013154	PPP2R5E	-
AY189737	OVGP1	+	AF020351	NDUFS4	+	AK055139	PTK2	-
AB014608	PARC	+	BC013789	NHLH1	+	AF218026	PTOV1	-
AL133335	PFON4	+		Nuclear Factor 1A	+	AF008591	RAC3	-
AJ419231	PHC2	-	BC011539	ORC1L	+	AL701206	RARG	+
AF006501	POLR2P	+	AB014887	ORM1	+	AF127761	RBM8A	-
AK095191	POU6F1	-	BC006268	PEX7	+	AF155595	RCOR	+
AF045569	PRKCH	+	AK093558	PFDN1	+	AB007148	RPS3A	-
NM_006256	PRKCL2	+	AL133335	PFDN4	+	BC007102	RQCD1	-
AF007157	PRNPIP	-	BC009899	PIK3R4	+	BC005927	SERPINE1	+
	N-Cym	+	BC037246	PNMT	+	AB007897	SETBP1	+
AK074531	PRR3	-	AF055028	POLR2B	+	BC009362	SETDB1	+
AF332577	PSMA6	+	BC031043	PRH1	+	AF029081	SFN	+
AF000231	RAB11A	-	AB026491	PRKCABP	+	AF368279	SGTA	-
AF125393	RAB27A	+	AK074531	PRR3	-	AK000416	SLC16A5	+
BC002585	RAB7L1	+	AF332577	PSMA6	+	AF078544	SLC25A14	+
D38076	RANBP1	-	AK023775	PTPRF	-	AK127096	SLC30A3	+
AB112074	RBBP6	+	AF263016	PTPRR	-	AY142112	SLC4A3	+
BC007102	RQCD1	-	BC001390	QP-C	+	BC009409	TACSTD2	+
AF072825	RREB1	+	BC015460	QPCT	+	AB006630	TCF20	-
AC004381	SAH	+	AF000231	RAB11A	-	AF142482	TEAD3	+
AF015224	SCGB2A2	+	AK055170	RAE1	+	BC000866	TIMP1	+
AF029081	SFN	+	AF127761	RBM8A	+	BC029516	TNP1	+
AK127319	SLC16A3	-	AF155595	RCOR	-	AF038009	TPST1	-
BC041164	SMPD1	-	BX537448	SEC14L1	-	AY245544	TRB2	+
AB046845	SMURF1	-	AF153609	SGK	-	AF104927	TTLL1	+
AB030036	ST14	+	AF078544	SLC25A14	+	BX537824	TXNIP	+
AF070532	SUPT6H	-	BC009409	TACSTD2	+	AB002155	UPK1B	+
AJ549245	TAF1	+	AF142482	TEAD3	-	AF122922	WIF1	
BC029891	TFEC	+	BC000866	TIMP1	+			
BC000866	TIMP1	+	AF017146	TOP3B	+			
AF139460	ZNF288	+	BC016804	TRAM2	-			
			BC014243	TYK2	-			
			AB028980	USP24	+			
			AB017103	YWHAE	-			

Abstract

The present invention provides a method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject (such as endometrium, peritoneal fluid, and/or smooth muscle cells); (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest; and (c) correlating the expression of the gene(s) with the presence or absence of the fibrotic disorder in the subject. The present invention also provides a method and compositions for modulating the expression of genes that are differentially expressed in fibrotic tissues, compared to normal tissues. Restoration of gene expression to levels associated with normal tissue is expected to ameliorate at least some of the symptoms of the fibrotic disorder. This method includes the step of contacting the tissue with an agent that modulates expression of one or more differentially expressed genes in the tissue.

Figure 1:

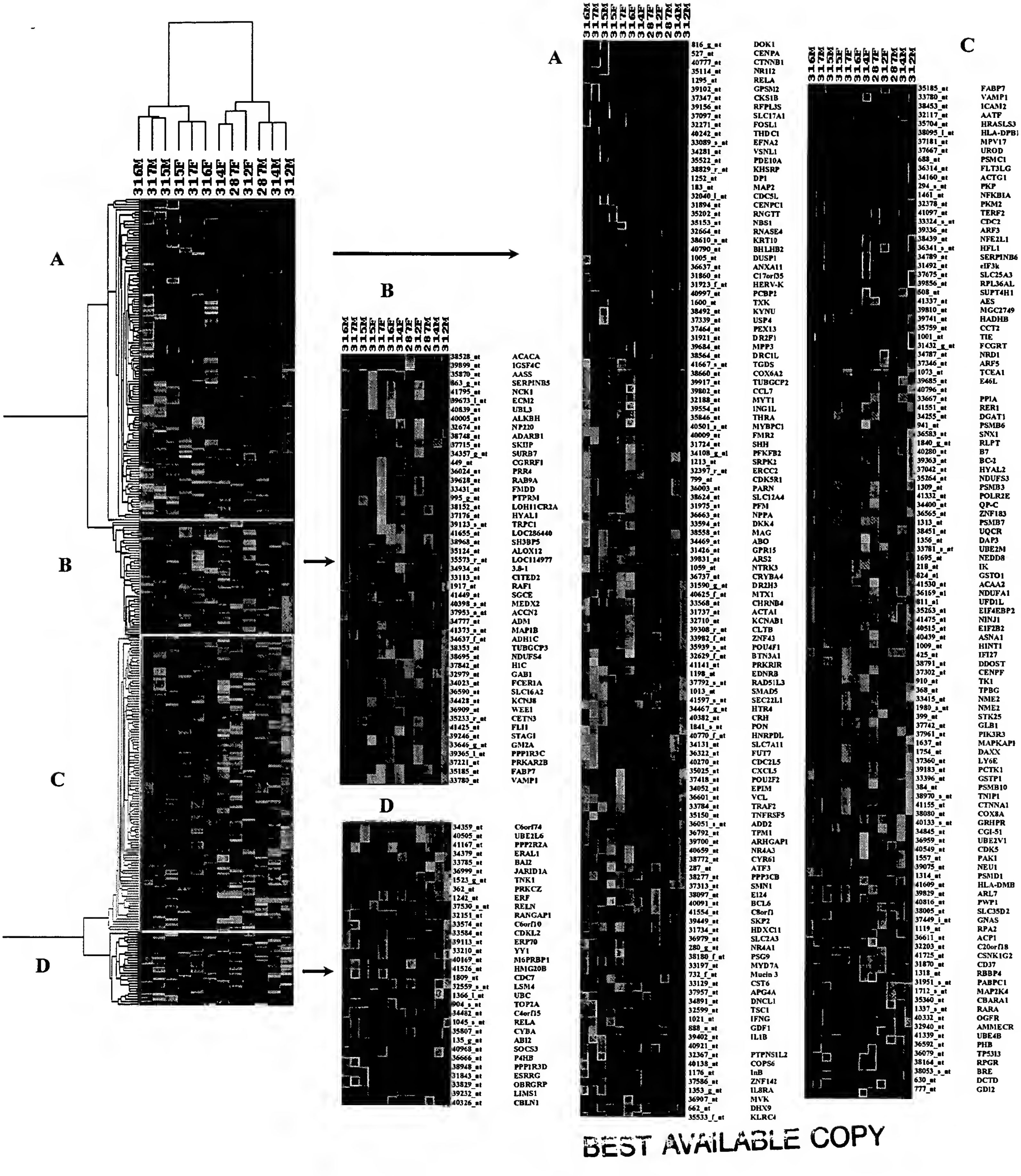
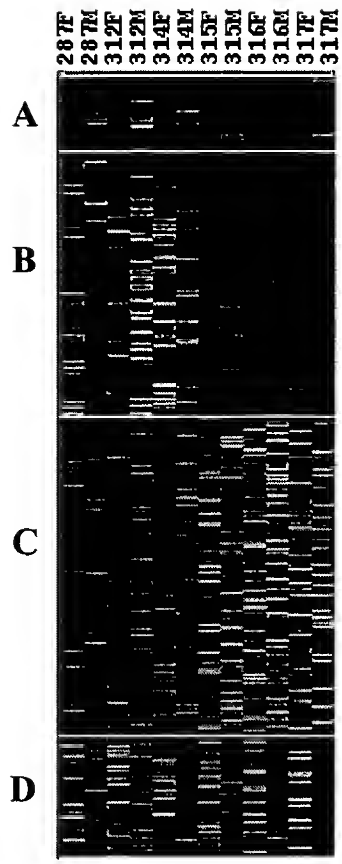


Figure 2: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287M
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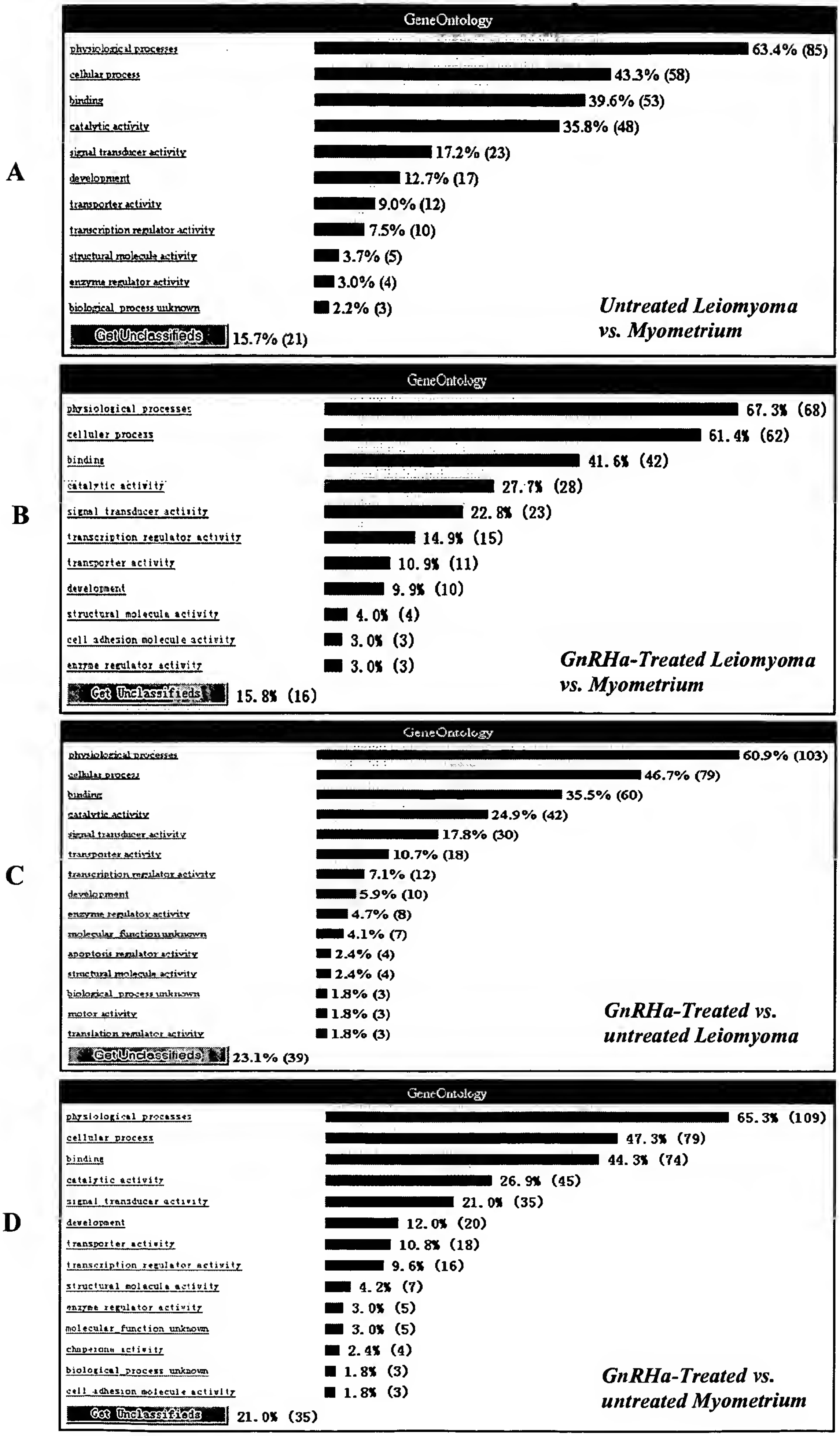
317M
A
B
C
D

Figure 3: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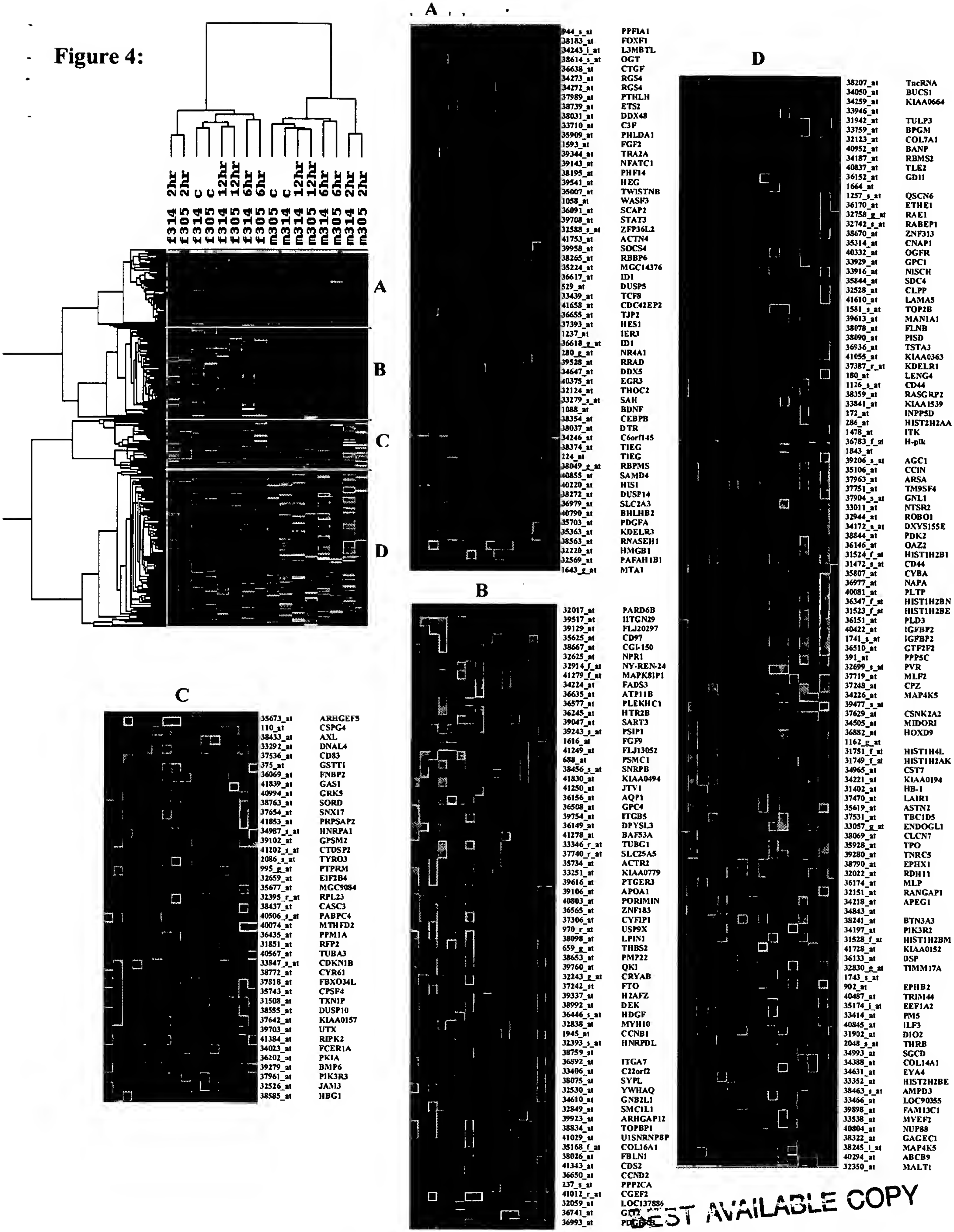
GeneOntology
physiological processes 63.4% (85)
cellular process 43.3% (58)
binding 39.6% (53)
catalytic activity 35.8% (48)
signal transducer activity 17.2% (23)
development 12.7% (17)
transporter activity 9.0% (12)
transcription regulator activity 7.5% (10)
structural molecule activity 3.7% (5)
enzyme regulator activity 3.0% (4)
biological process unknown 2.2% (3)
Get Unclassifieds 15.7% (21)
Untreated Leiomyoma vs. Myometrium
B
GeneOntology
physiological processes 67.3% (68)
cellular process 61.4% (62)
binding 41.6% (42)
catalytic activity 27.7% (28)
signal transducer activity 22.8% (23)
transcription regulator activity 14.9% (15)
transporter activity 10.9% (11)
development 9.9% (10)
structural molecule activity 4.0% (4)
cell adhesion molecule activity 3.0% (3)
enzyme regulator activity 3.0% (3)
Get Unclassifieds 15.8% (16)
GnRHa-Treated Leiomyoma vs. Myometrium
C
GeneOntology
physiological processes 60.9% (103)
cellular process 46.7% (79)
binding 35.5% (60)
catalytic activity 24.9% (42)
signal transducer activity 17.8% (30)
transporter activity 10.7% (18)
transcription regulator activity 7.1% (12)
development 5.9% (10)
enzyme regulator activity 4.7% (8)
molecular function unknown 4.1% (7)
apoptosis regulator activity 2.4% (4)
structural molecule activity 2.4% (4)
biological process unknown 1.8% (3)
motor activity 1.8% (3)
translation regulator activity 1.8% (3)
Get Unclassifieds 23.1% (39)
GnRHa-Treated vs. untreated Leiomyoma
D
GeneOntology
physiological processes 65.3% (109)
cellular process 47.3% (79)
binding 44.3% (74)
catalytic activity 26.9% (45)
signal transducer activity 21.0% (35)
development 12.0% (20)
transporter activity 10.8% (18)
transcription regulator activity 9.6% (16)
structural molecule activity 4.2% (7)
enzyme regulator activity 3.0% (5)
molecular function unknown 3.0% (5)
chaperone activity 2.4% (4)
biological process unknown 1.8% (3)
cell adhesion molecule activity 1.8% (3)
Get Unclassifieds 21.0% (35)
GnRHa-Treated vs. untreated Myometrium

Figure 4:



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f305 2hr
f314 c
f305 c
f314 12hr
f305 12hr
f314 6hr
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m305 c
m314 c
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m314 6hr
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m305 2hr
A
B
C
D
A
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38183_at FOXF1
34243_i_at L3MBTL
38614_s_at OGT
36638_at CTGF
34273_at RGS4
34272_at RGS4
37989_at PTHLH
38739_at ETS2
38031_at DDX48
33710_at C3F
35909_at PHLDA1
1593_at FGF2
39344_at TRA2A
39143_at NFATC1
38195_at PHF14
39541_at HEG
35007_at TWISTNB
1058_at WASF3
36091_at SCAP2
39708_at STAT3
32588_s_at ZFP36L2
41753_at ACTN4
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38265_at RBBP6
35224_at MGC14376
36617_at ID1
529_at DUSP5
33439_at TCF8
41658_at CDC42EP2
36655_at TJP2
37393_at HES1
1237_at IER3
36618_g_at ID1
280_g_at NR4A1
39528_at RRAD
34647_at DDX5
40375_at EGR3
32124_at THOC2
33279_s_at SAH
1088_at BDNF
38354_at CEBPB
38037_at DTR
34246_at C6orf145
38374_at TIEG
224_at TIEG
38049_g_at RBPMS
40855_at SAMD4
40220_at HIS1
38272_at DUSP14
36979_at SLC2A3
40790_at BHLHB2
35703_at PDGFA
35363_at KDELR3
38563_at RNASEH1
32220_at HMGB1
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38667_at CGI-150
32625_at NPR1
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41279_f_at MAPK8IP1
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36635_at ATP11B
36577_at PLEKHC1
36245_at HTR2B
39047_at SART3
39243_s_at PSIP1
1616_at FGF9
41249_at FLJ13052
688_at PSMC1
38456_s_at SNRPB
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41250_at JTV1
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36508_at GPC4
39754_at ITGB5
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41278_at BAF53A
33346_r_at TUBG1
37740_r_at SLC25A5
35734_at ACTR2
33251_at KIAA0779
39616_at PTGER3
39106_at APOA1
40803_at PORIMIN
36565_at ZNF183
37306_at CYFIP1
970_r_at USP9X
38098_at LPIN1
659_g_at THBS2
38653_at PMP22
39760_at QKI
32243_g_at CRYAB
37242_at FTO
39337_at H2AFZ
38992_at DEK
36446_s_at HDGF
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1945_at CCNB1
32393_s_at HNRPDL
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36892_at ITGA7
33406_at C22orf2
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32530_at YWHAQ
34610_at GNB2L1
32849_at SMC1L1
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110_at CSPG4
38433_at AXL
33292_at DNAL4
37536_at CD83
375_at GSTT1
36069_at FNBP2
41839_at GAS1
40994_at GRK5
38763_at SORD
37654_at SNX17
41853_at PRPSAP2
34987_s_at HNRPA1
39102_at GPSM2
41202_s_at CTDSP2
2086_s_at TYRO3
995_g_at PTPRM
32659_at EIF2B4
35677_at MGC9084
32395_r_at RPL23
38437_at CASC3
40506_s_at PABPC4
40074_at MTHFD2
36435_at PPM1A
31851_at RFP2
40567_at TUBA3
33847_s_at CDKN1B
38772_at CYR61
37818_at FBXO34L
35743_at CPSF4
31508_at TXNIP
38555_at DUSP10
37642_at KIAA0157
39703_at UTX
41384_at RIPK2
34023_at FCER1A
36202_at PKIA
39279_at BMP6
37961_at PIK3R3
32526_at JAM3
38585_at HBG1
D
38207_at TncRNA
34050_at BUCS1
34259_at KIAA0664
33946_at
31942_at TULP3
33759_at BPGM
32123_at COL7A1
40952_at BANP
34187_at RBMS2
40837_at TLE2
36152_at GDI1
1664_at
1257_s_at QSCN6
36170_at ETHE1
32758_g_at RAE1
32742_s_at RABEP1
38670_at ZNF313
35314_at CNAP1
40332_at OGFR
33929_at GPC1
33916_at NISCH
35844_at SDC4
32528_at CLPP
41610_at LAMA5
1581_s_at TOP2B
39613_at MAN1A1
38078_at FLNB
38090_at PISD
36936_at TSTA3
41055_at KIAA0363
37387_r_at KDELR1
180_at LENG4
1126_s_at CD44
38359_at RASGRP2
33841_at KIAA1539
172_at INPP5D
286_at HIST2H2AA
1478_at ITK
36783_f_at H-plk
1843_at
39206_s_at AGC1
35106_at CCIN
37963_at ARSA
37751_at TM9SF4
37904_s_at GNL1
33011_at NTSR2
32944_at ROBO1
34172_s_at DXYS155E
38844_at PDK2
36146_at OAZ2
31524_f_at HIST1H2BI
31472_s_at CD44
35807_at CYBA
36977_at NAPA
40081_at PLTP
36347_f_at HIST1H2BN
31523_f_at HIST1H2BE
36151_at PLD3
40422_at IGFBP2
1741_s_at IGFBP2
36510_at GTF2F2
391_at PPP5C
32699_s_at PVR
37719_at MLF2
37248_at CPZ
34226_at MAP4K5
39477_s_at
37629_at CSNK2A2
34505_at MIDORI
36882_at HOXD9
1162_g_at
31751_f_at HIST1H4L
31749_f_at HIST1H2AK
34965_at CST7
34221_at KIAA0194
31402_at HB-1
37470_at LAIR1
35619_at ASTN2
37531_at TBC1D5
33057_g_at ENDOGL1
38069_at CLCN7
35928_at TPO
39280_at TNRC5
38790_at EPHX1
32022_at RDH11
36174_at MLP
32151_at RANGAP1
34218_at APEG1
34843_at
38241_at BTN3A3
34197_at PIK3R2
31528_f_at HIST1H2BM
41728_at KIAA0152
36133_at DSP
32830_g_at TIMM17A
1743_s_at
902_at EPHB2
40487_at TRIM14
35174_i_at EEF1A2
33414_at PMS
40845_at ILF3
31902_at DIO2
2048_s_at THRB
34993_at SGCD
34388_at COL14A1
34631_at EYA4
33352_at HIST2H2BE
38463_s_at AMPD3
33466_at LOC90355
39898_at FAM13C1
33538_at MYEF2
40804_at NUP88
38322_at GAGEC1
38245_i_at MAP4K5
40294_at ABCB9
32350_at MALT1

Figure 5:

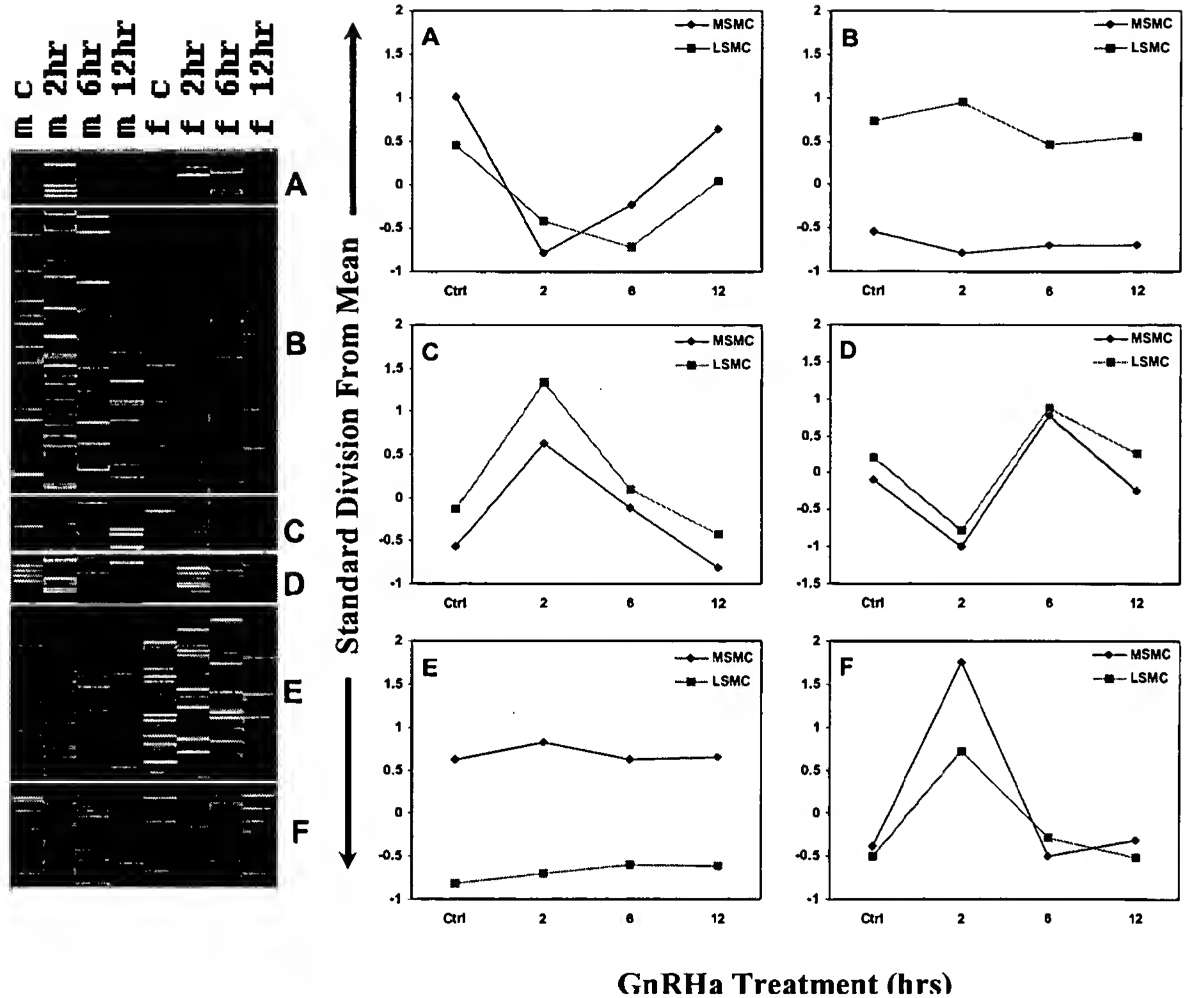
m C
m 2hr
m 6hr
m 12hr
f C
f 2hr
f 6hr
f 12hr
A
B
C
D
E
F
Standard Division From Mean
MSMC
LSMC
Ctrl
2
6
12
GnRHa Treatment (hrs)

Figure 6:

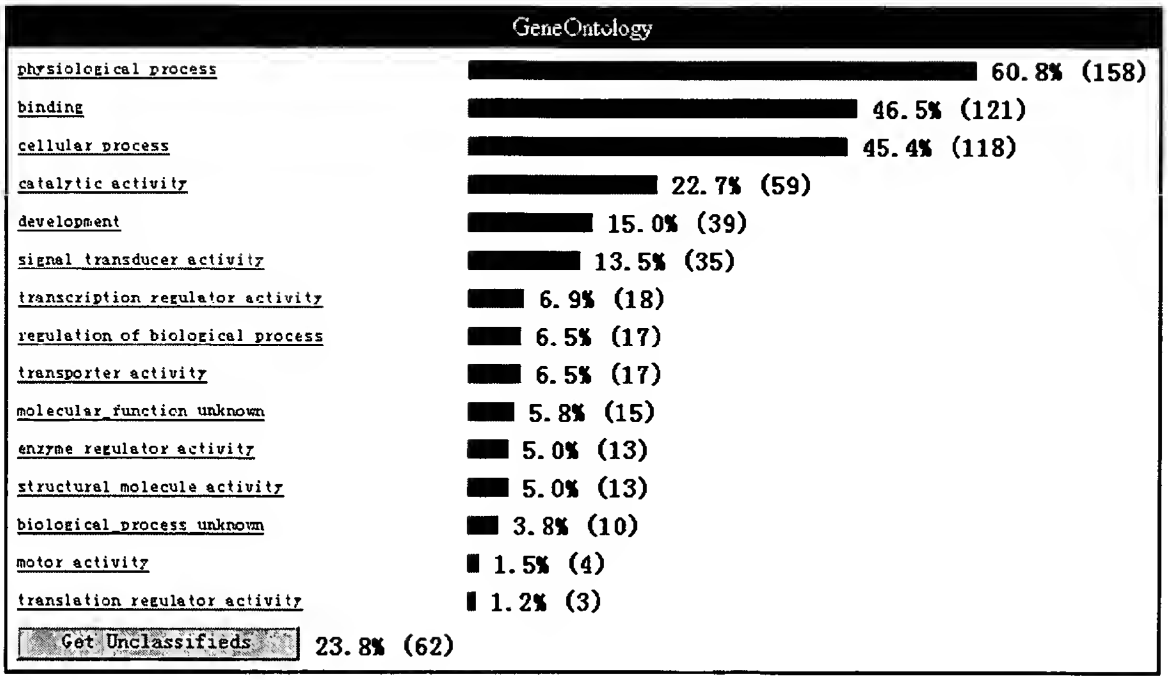
GeneOntology
physiological process 60.8% (158)
binding 46.5% (121)
cellular process 45.4% (118)
catalytic activity 22.7% (59)
development 15.0% (39)
signal transducer activity 13.5% (35)
transcription regulator activity 6.9% (18)
regulation of biological process 6.5% (17)
transporter activity 6.5% (17)
molecular_function unknown 5.8% (15)
enzyme regulator activity 5.0% (13)
structural molecule activity 5.0% (13)
biological_process unknown 3.8% (10)
motor activity 1.5% (4)
translation regulator activity 1.2% (3)
Get Unclassifieds 23.8% (62)

Figure 7:

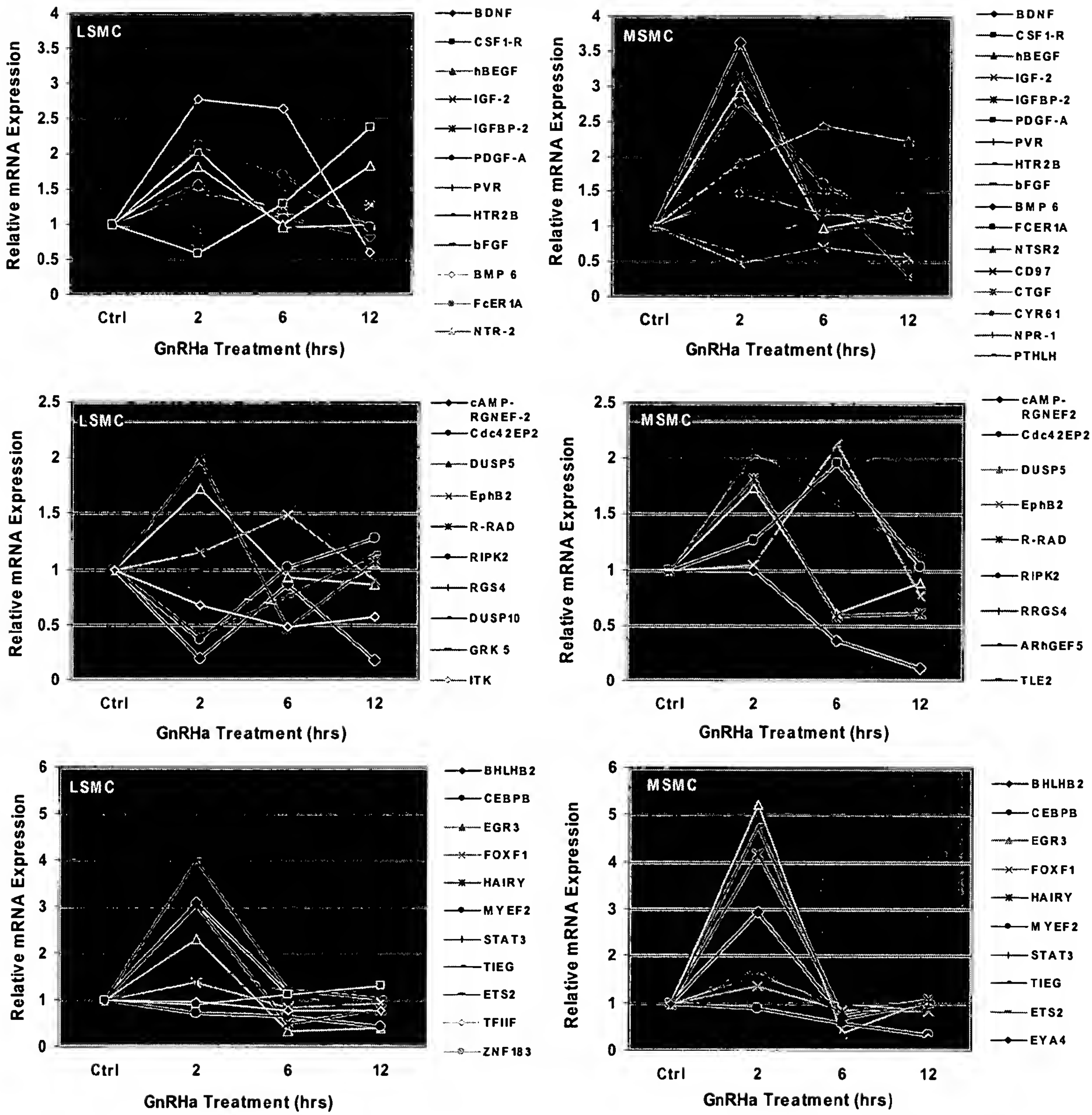

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Figure 7 (Cont):

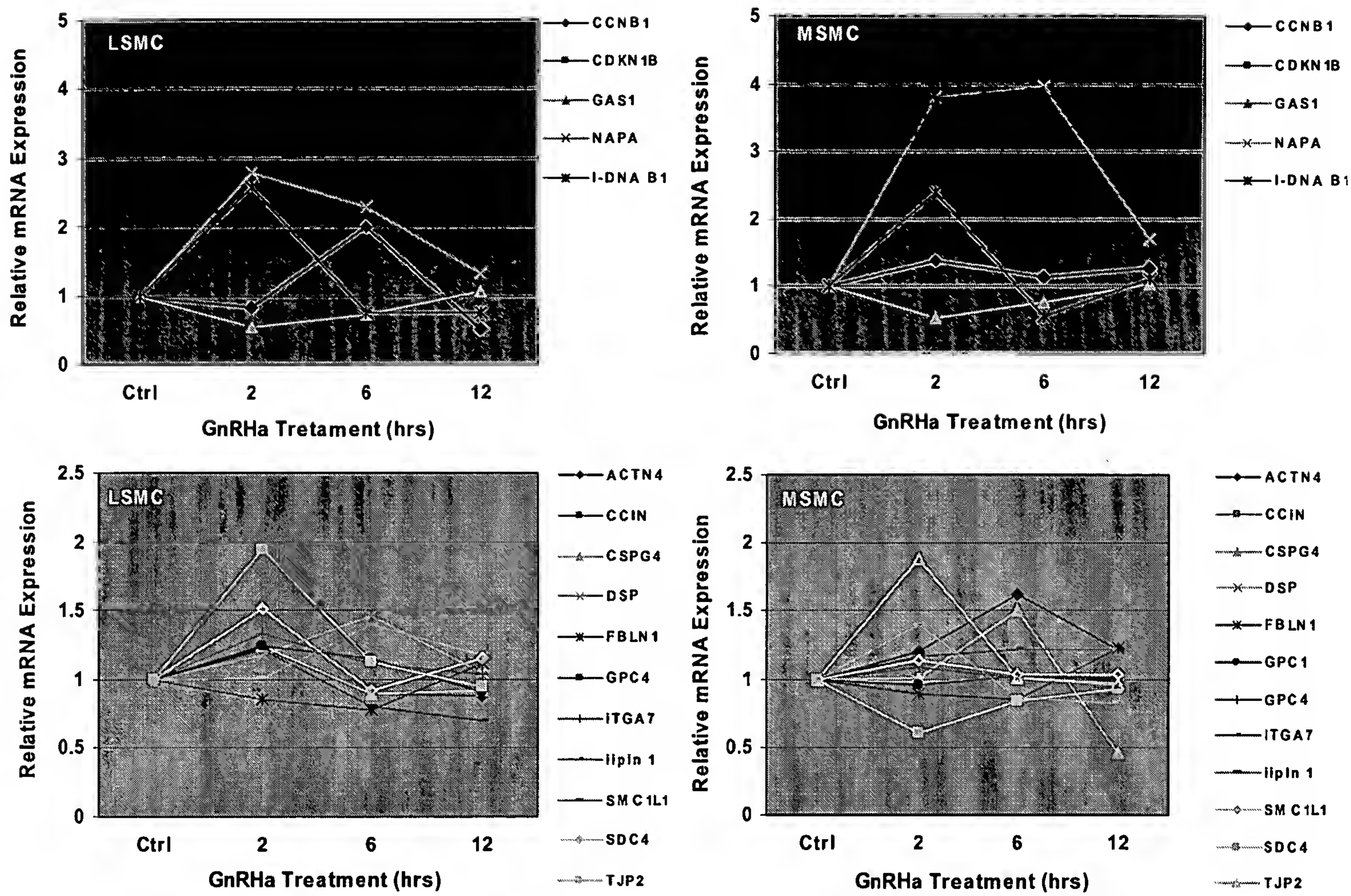

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Figure 8:

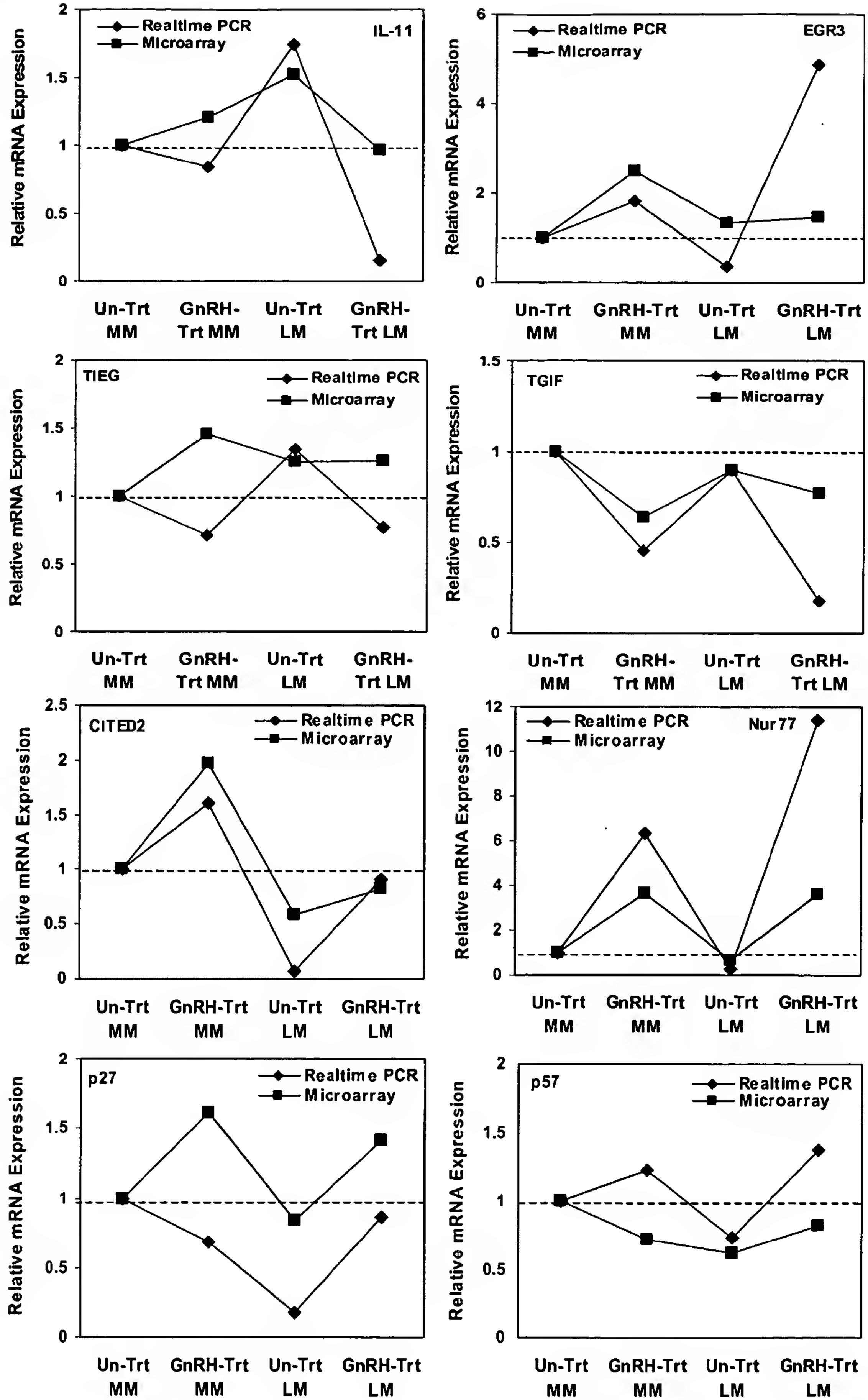
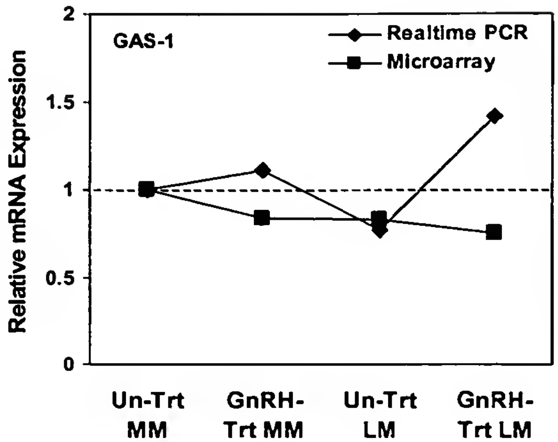

Realtime PCR
Microarray
IL-11
EGR3
TIEG
TGIF
CITED2
Nur77
p27
p57
Relative mRNA Expression
Un-Trt MM
GnRH-Trt MM
Un-Trt LM
GnRH-Trt LM
0
0.5
1
1.5
2
2.5
4
6
8
10
12

Figure 8 (Cont):



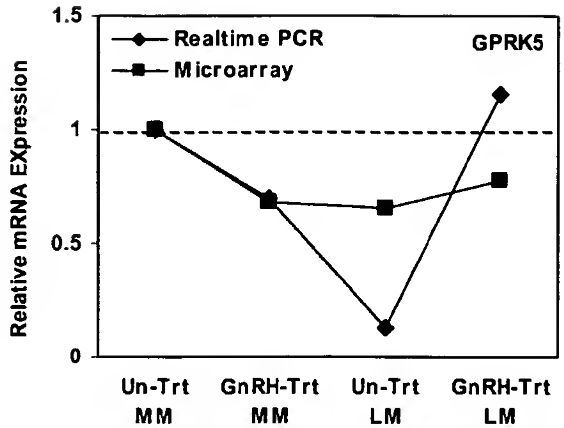
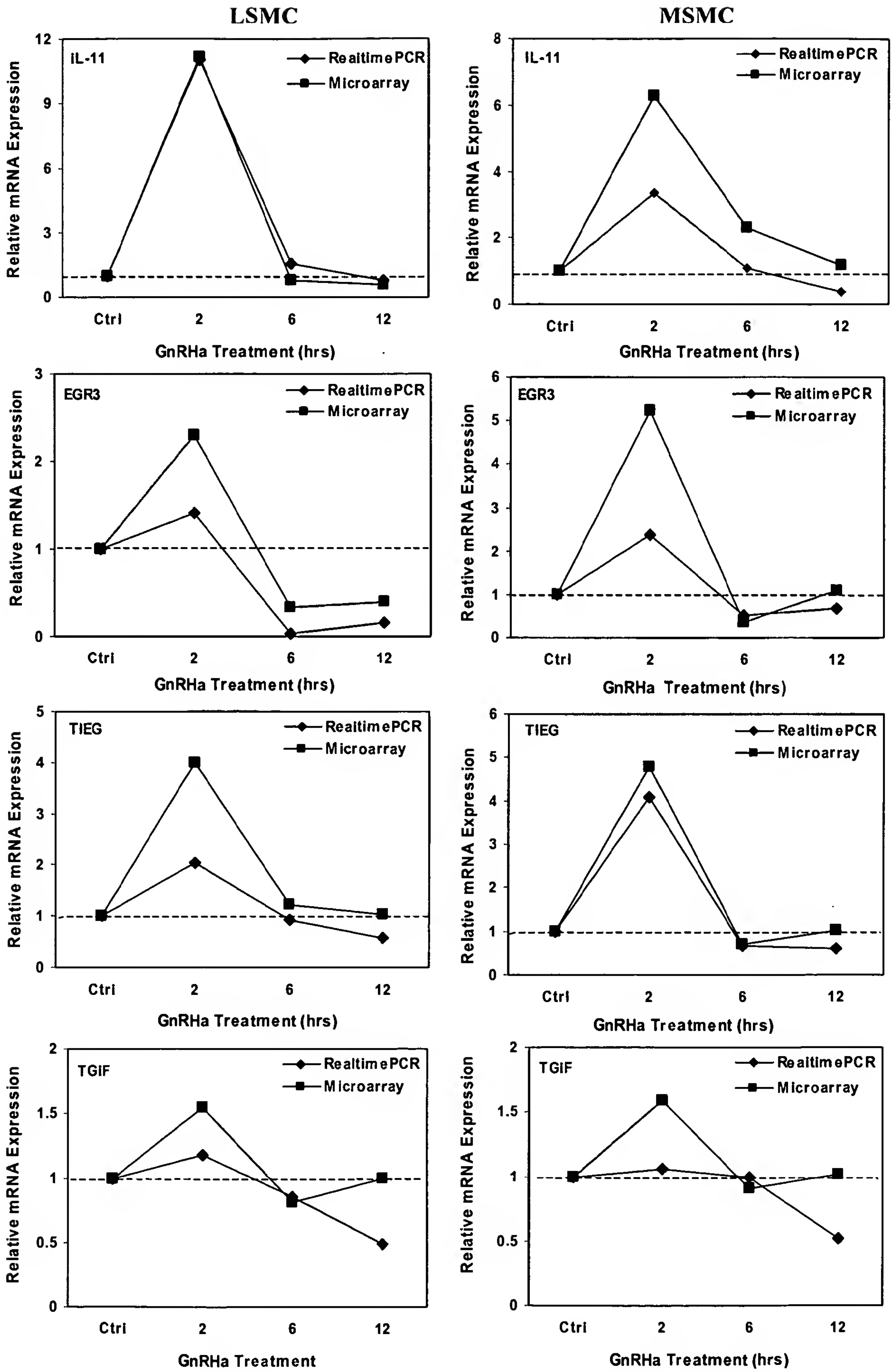


Figure 9:



LSMC
MSMC
IL-11
EGR3
TIEG
TGIF
RealtimePCR
Microarray
Relative mRNA Expression
GnRHa Treatment (hrs)
GnRHa Treatment
Ctrl
2
6
12

Figure 9 (Cont):

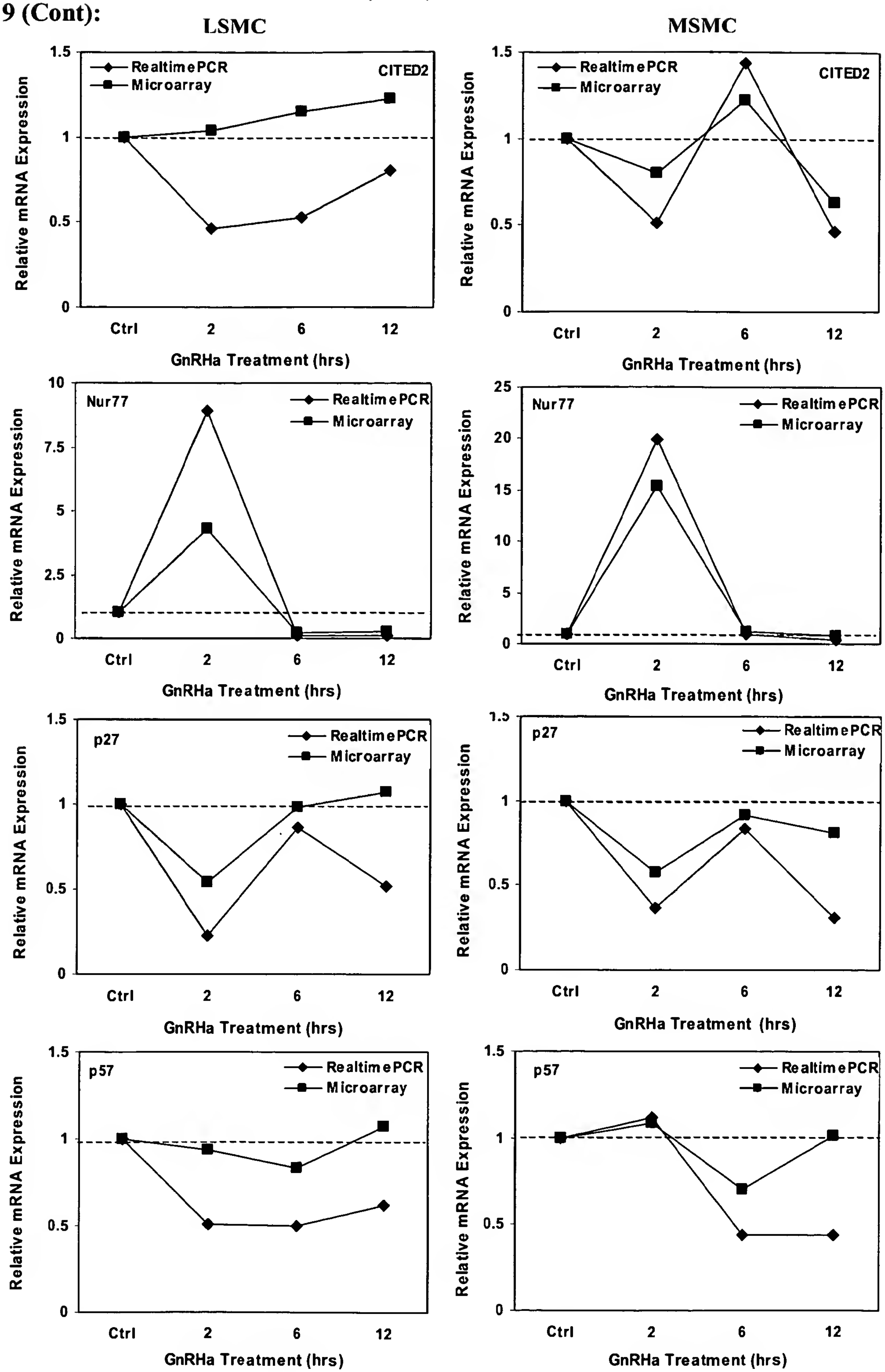


Figure 9 (Cont):

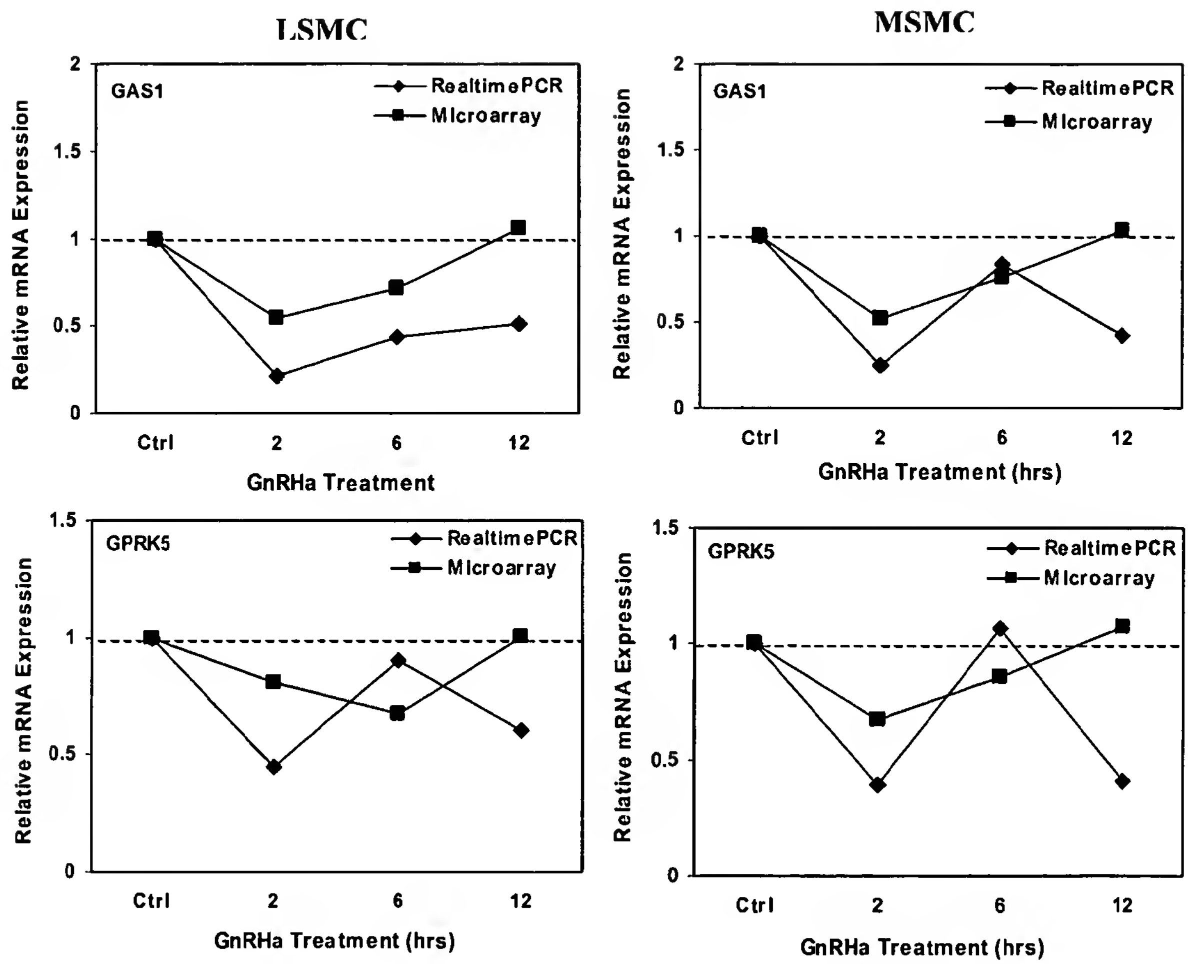


Figure 10

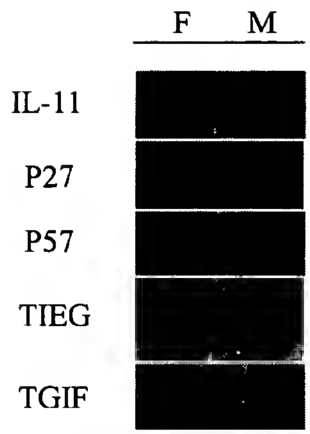


Figure 11:

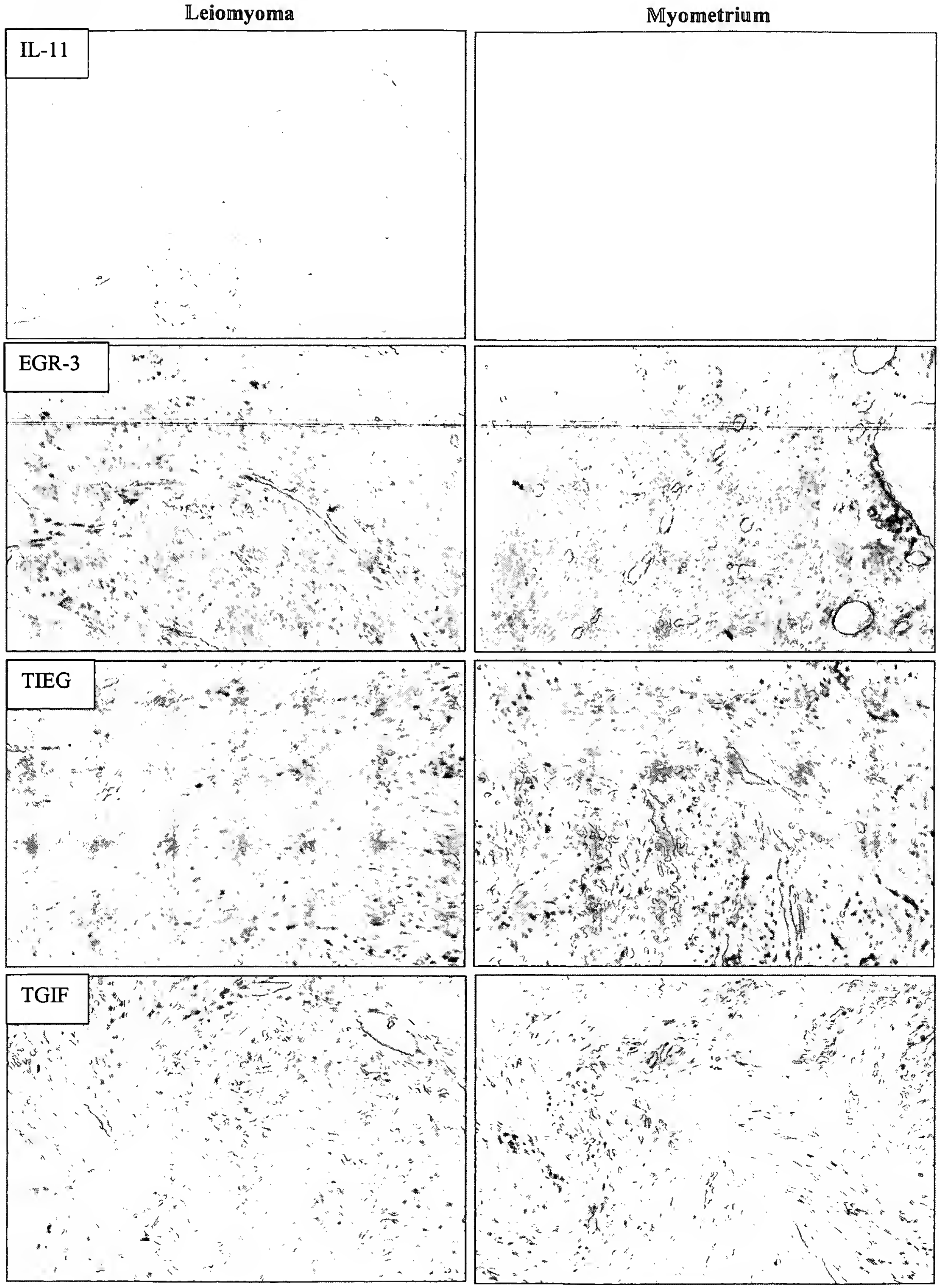


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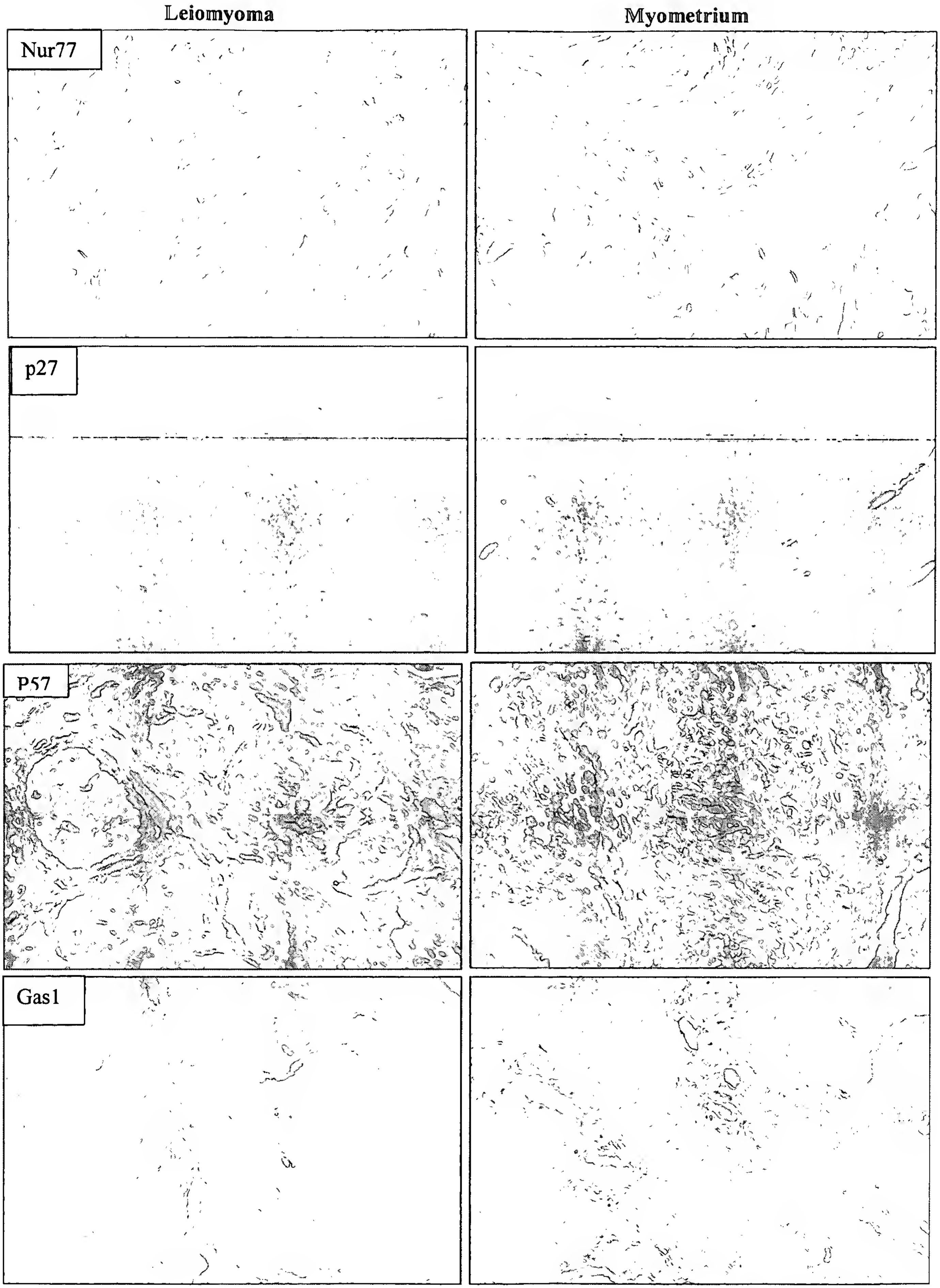
Figure 11 (Cont):



BEST AVAILABLE COPY

Figure 11 (Cont):

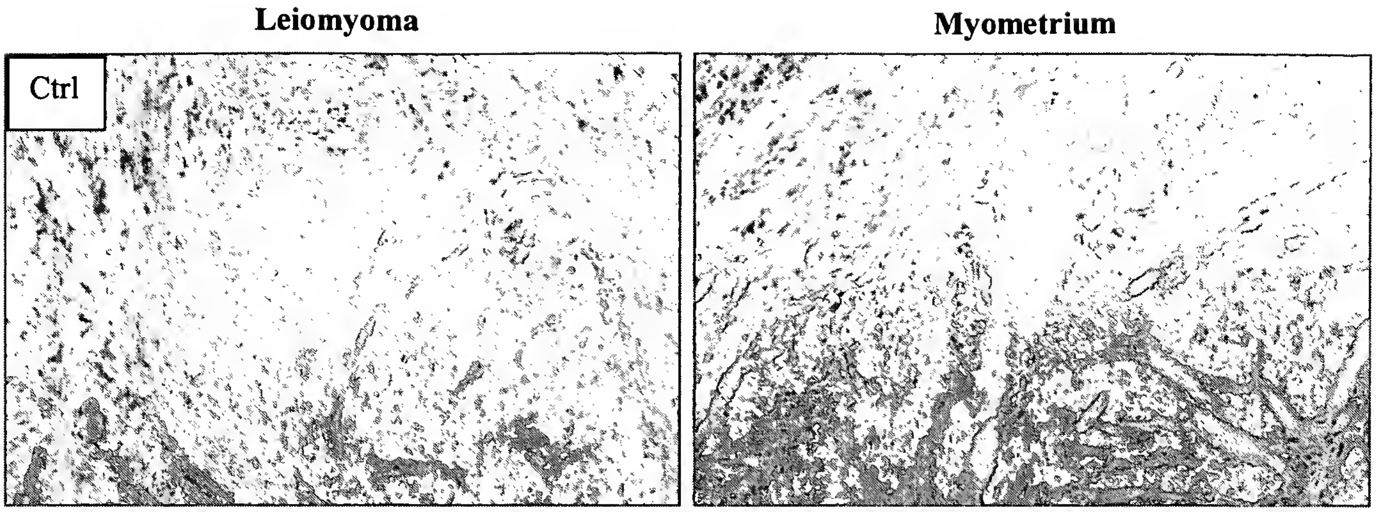
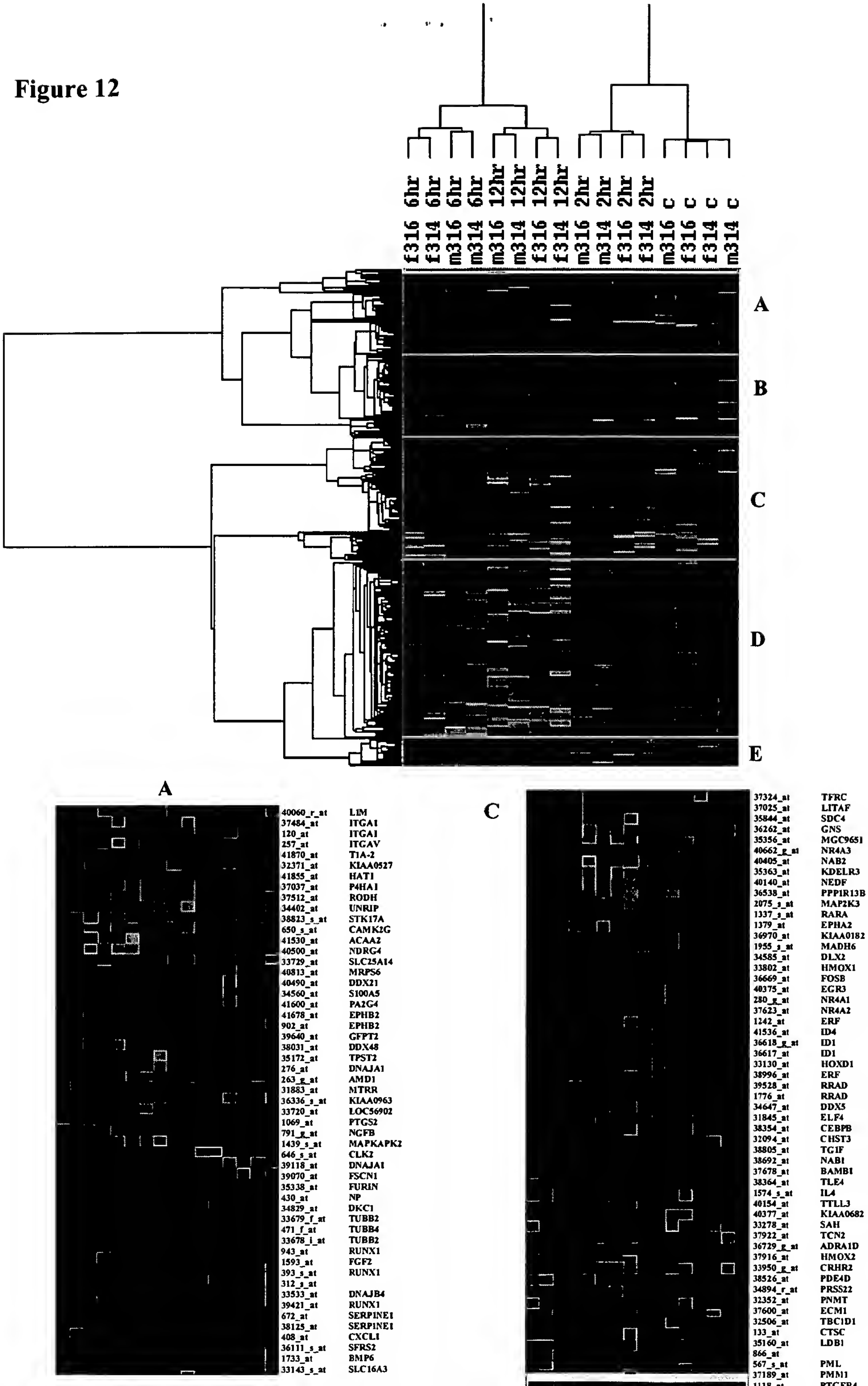


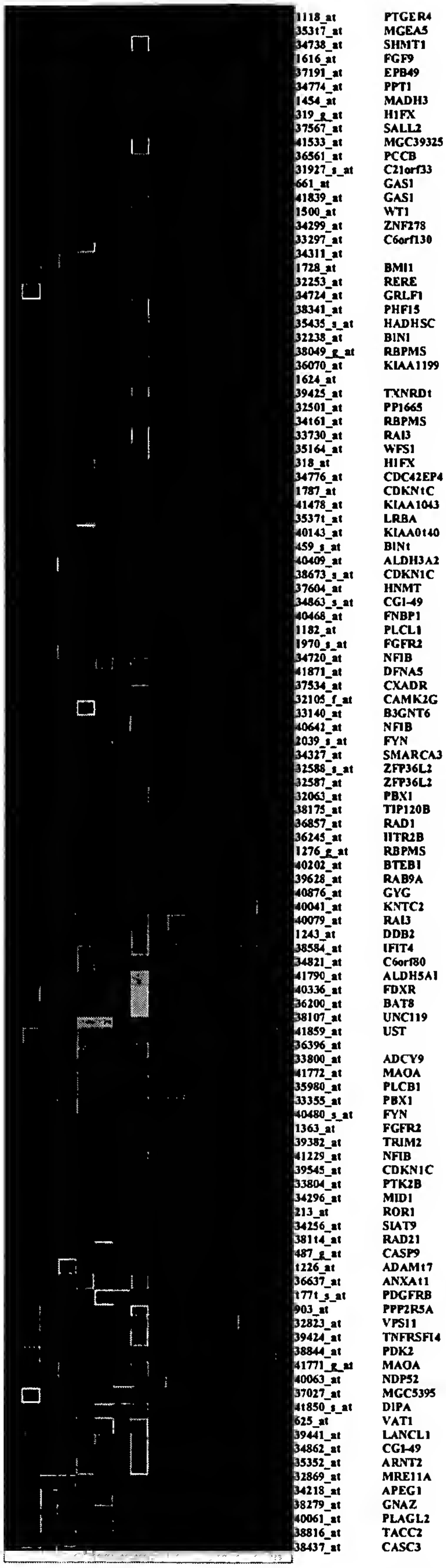
Figure 12



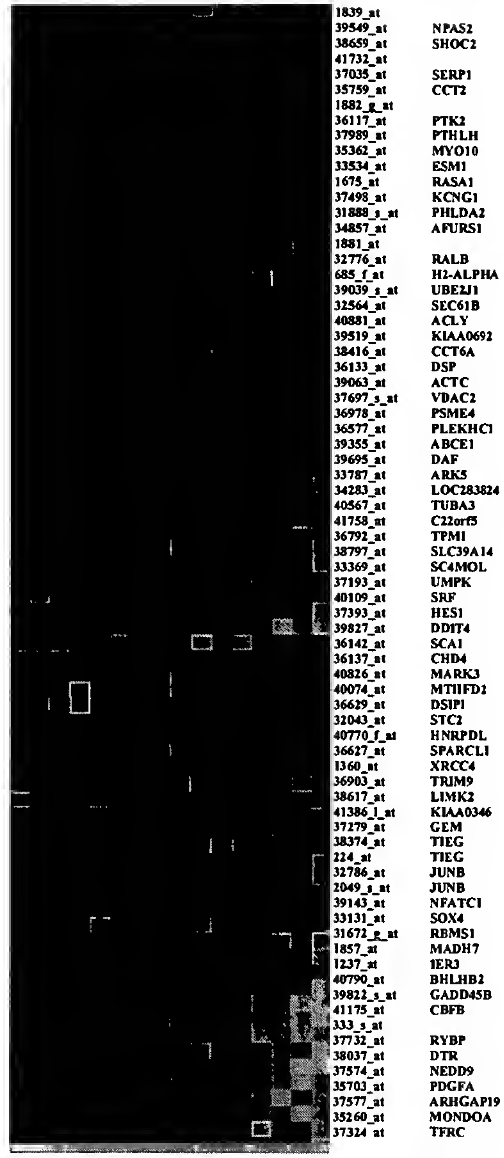
f316 6hr
f314 6hr
m316 6hr
m314 6hr
m316 12hr
m314 12hr
f316 12hr
f314 12hr
m316 2hr
m314 2hr
f316 2hr
f314 2hr
m316 c
f316 c
f314 c
m314 c
A
B
C
D
E
A
40060_r_at LIM
37484_at ITGA1
120_at ITGA1
257_at ITGAV
41870_at T1A-2
32371_at KIAA0527
41855_at HAT1
37037_at P4HA1
37512_at RODH
34402_at UNRIP
38823_s_at STK17A
650_s_at CAMK2G
41530_at ACAA2
40500_at NDRG4
33729_at SLC25A14
40813_at MRPS6
40490_at DDX21
34560_at S100A5
41600_at PA2G4
41678_at EPHB2
902_at EPHB2
39640_at GFPT2
38031_at DDX48
35172_at TPST2
276_at DNAJA1
263_g_at AMD1
31883_at MTRR
36336_s_at KIAA0963
33720_at LOC56902
1069_at PTGS2
791_g_at NGFB
1439_s_at MAPKAPK2
646_s_at CLK2
39118_at DNAJA1
39070_at FSCN1
35338_at FURIN
430_at NP
34829_at DKC1
33679_f_at TUBB2
471_f_at TUBB4
33678_i_at TUBB2
943_at RUNX1
1593_at FGF2
393_s_at RUNX1
312_s_at
33533_at DNAJB4
39421_at RUNX1
672_at SERPINE1
38125_at SERPINE1
408_at CXCL1
36111_s_at SFRS2
1733_at BMP6
33143_s_at SLC16A3
C
37324_at TFRC
37025_at LITAF
35844_at SDC4
36262_at GNS
35356_at MGC9651
40662_g_at NR4A3
40405_at NAB2
35363_at KDELR3
40140_at NEDF
36538_at PPP1R13B
2075_s_at MAP2K3
1337_s_at RARA
1379_at EPHA2
36970_at KIAA0182
1955_s_at MADH6
34585_at DLX2
33802_at HMOX1
36669_at FOSB
40375_at EGR3
280_g_at NR4A1
37623_at NR4A2
1242_at ERF
41536_at ID4
36618_g_at ID1
36617_at ID1
33130_at HOXD1
38996_at ERF
39528_at RRAD
1776_at RRAD
34647_at DDX5
31845_at ELF4
38354_at CEBPB
32094_at CHST3
38805_at TG1F
38692_at NAB1
37678_at BAMB1
38364_at TLE4
1574_s_at IL4
40154_at TTLL3
40377_at KIAA0682
33278_at SAH
37922_at TCN2
36729_g_at ADRA1D
37916_at HMOX2
33950_g_at CRHR2
38526_at PDE4D
34894_r_at PRSS22
32352_at PNMT
37600_at ECM1
32506_at TBC1D1
133_at CTSC
35160_at LDB1
866_at
567_s_at PML
37189_at PMM1
1118_at PTGER4

Figure 12 (cont):

D



B



E

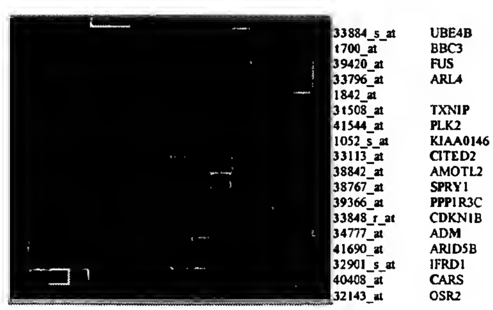


Figure 13

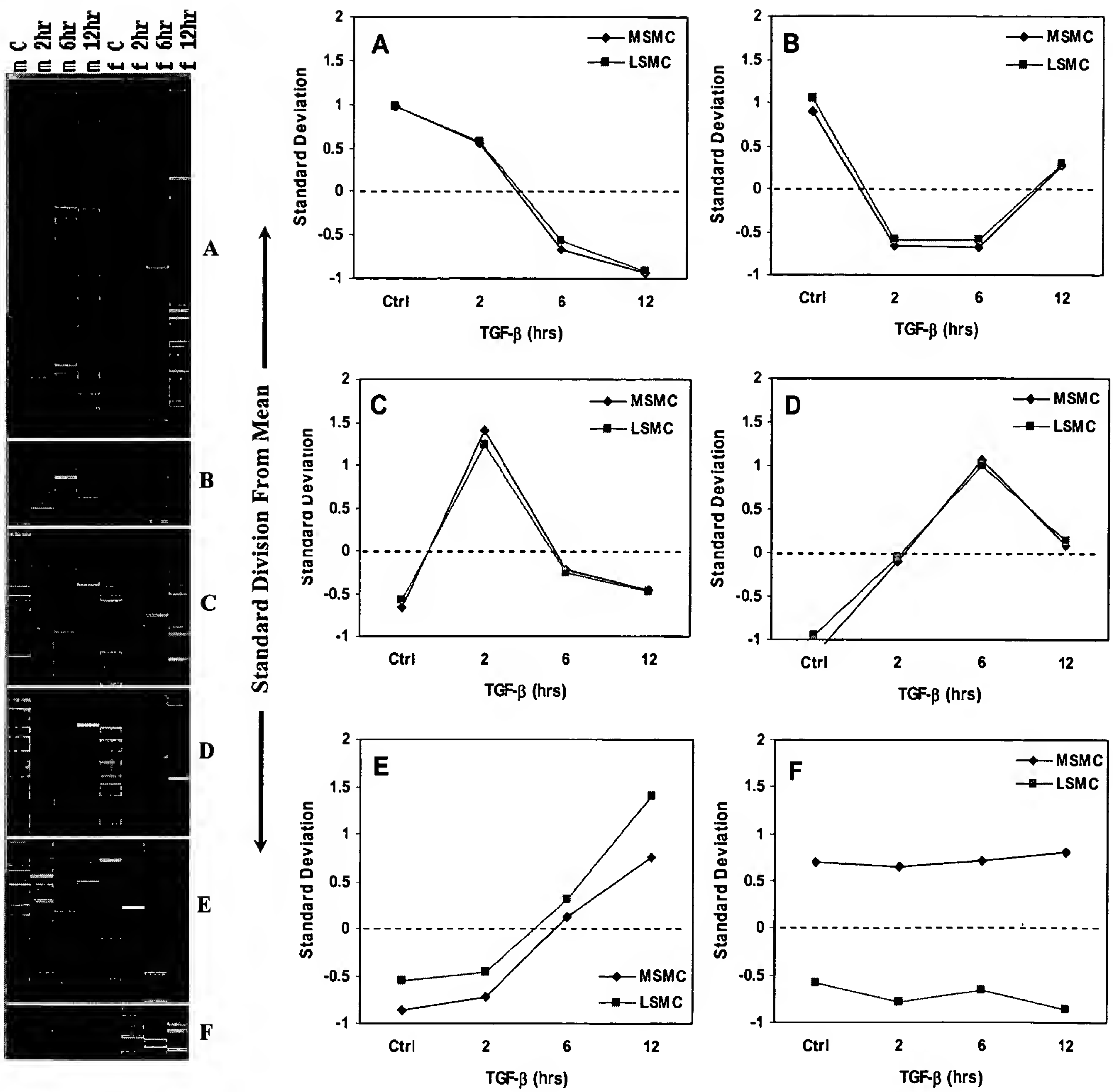

m C
m 2hr
m 6hr
m 12hr
f C
f 2hr
f 6hr
f 12hr
A
B
C
D
E
F
Standard Division From Mean
Standard Deviation
TGF-β (hrs)
Ctrl
2
6
12
MSMC
LSMC

Figure 14:

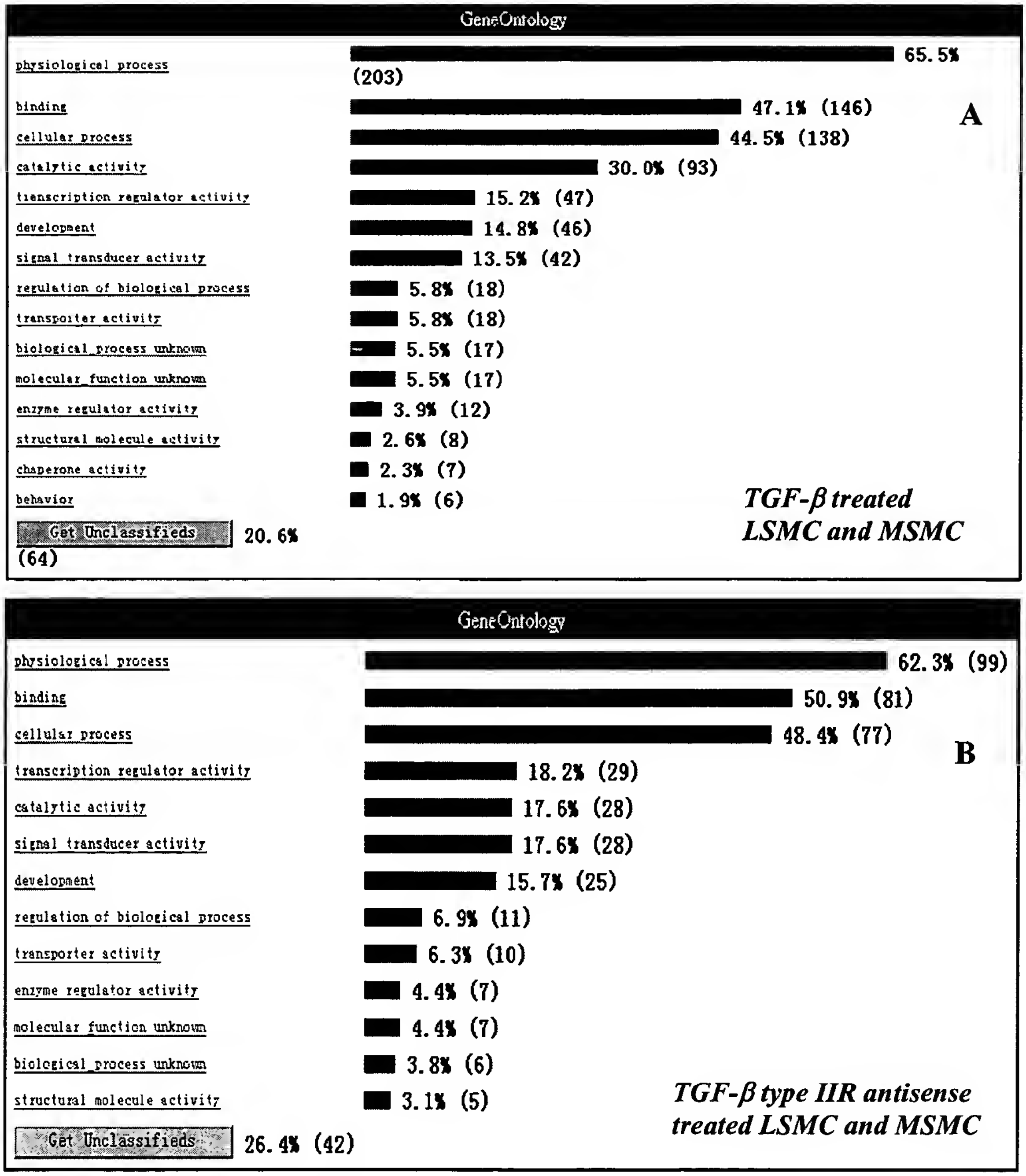

GeneOntology
physiological process
65.5% (203)
binding
47.1% (146)
cellular process
44.5% (138)
catalytic activity
30.0% (93)
transcription regulator activity
15.2% (47)
development
14.8% (46)
signal transducer activity
13.5% (42)
regulation of biological process
5.8% (18)
transporter activity
5.8% (18)
biological_process unknown
5.5% (17)
molecular_function unknown
5.5% (17)
enzyme regulator activity
3.9% (12)
structural molecule activity
2.6% (8)
chaperone activity
2.3% (7)
behavior
1.9% (6)
Get Unclassifieds
20.6% (64)
A
TGF-β treated LSMC and MSMC
GeneOntology
physiological process
62.3% (99)
binding
50.9% (81)
cellular process
48.4% (77)
transcription regulator activity
18.2% (29)
catalytic activity
17.6% (28)
signal transducer activity
17.6% (28)
development
15.7% (25)
regulation of biological process
6.9% (11)
transporter activity
6.3% (10)
enzyme regulator activity
4.4% (7)
molecular function unknown
4.4% (7)
biological_process unknown
3.8% (6)
structural molecule activity
3.1% (5)
Get Unclassifieds
26.4% (42)
B
TGF-β type IIR antisense treated LSMC and MSMC

Figure 15:

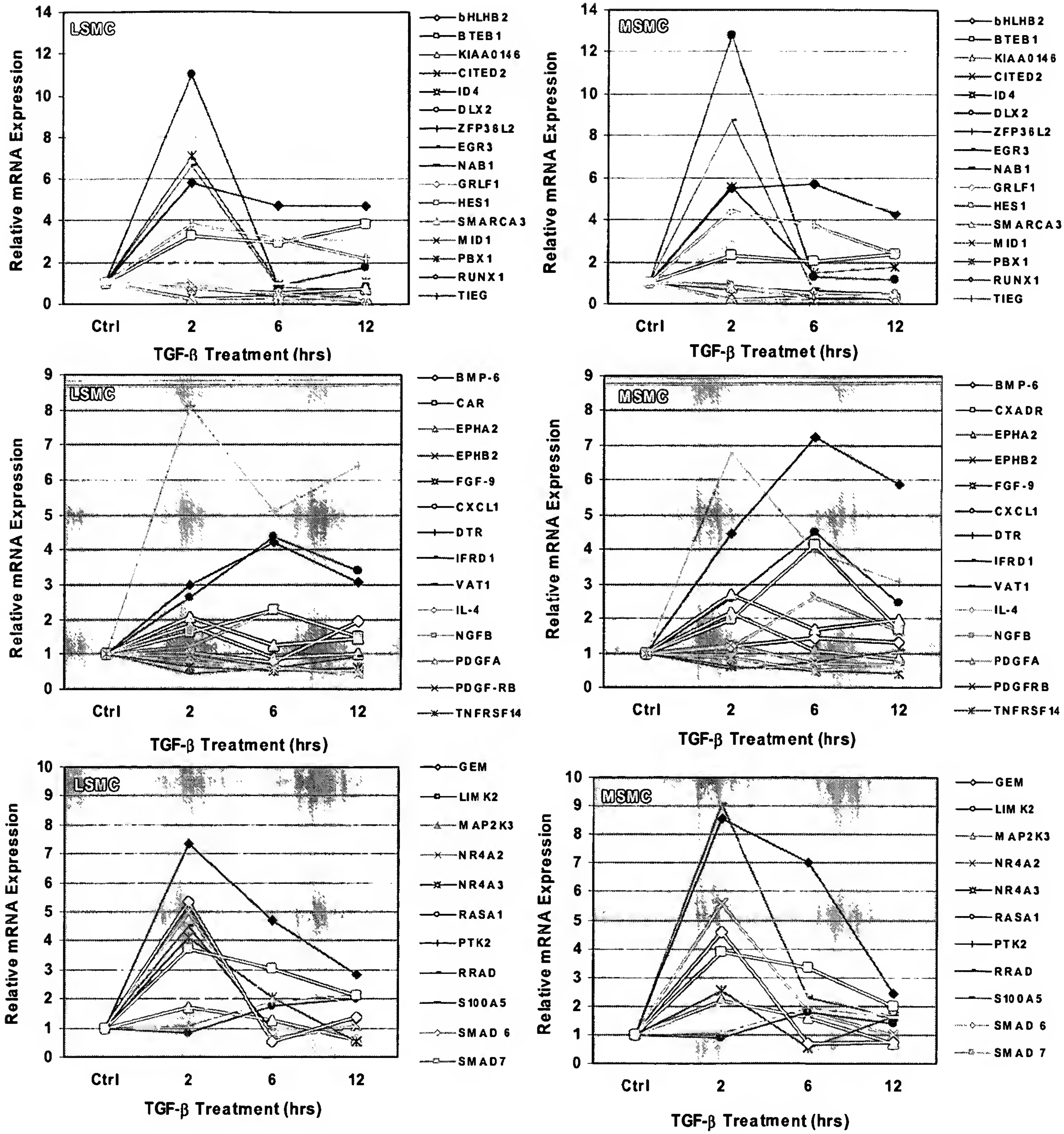


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Figure 15 (Cont):

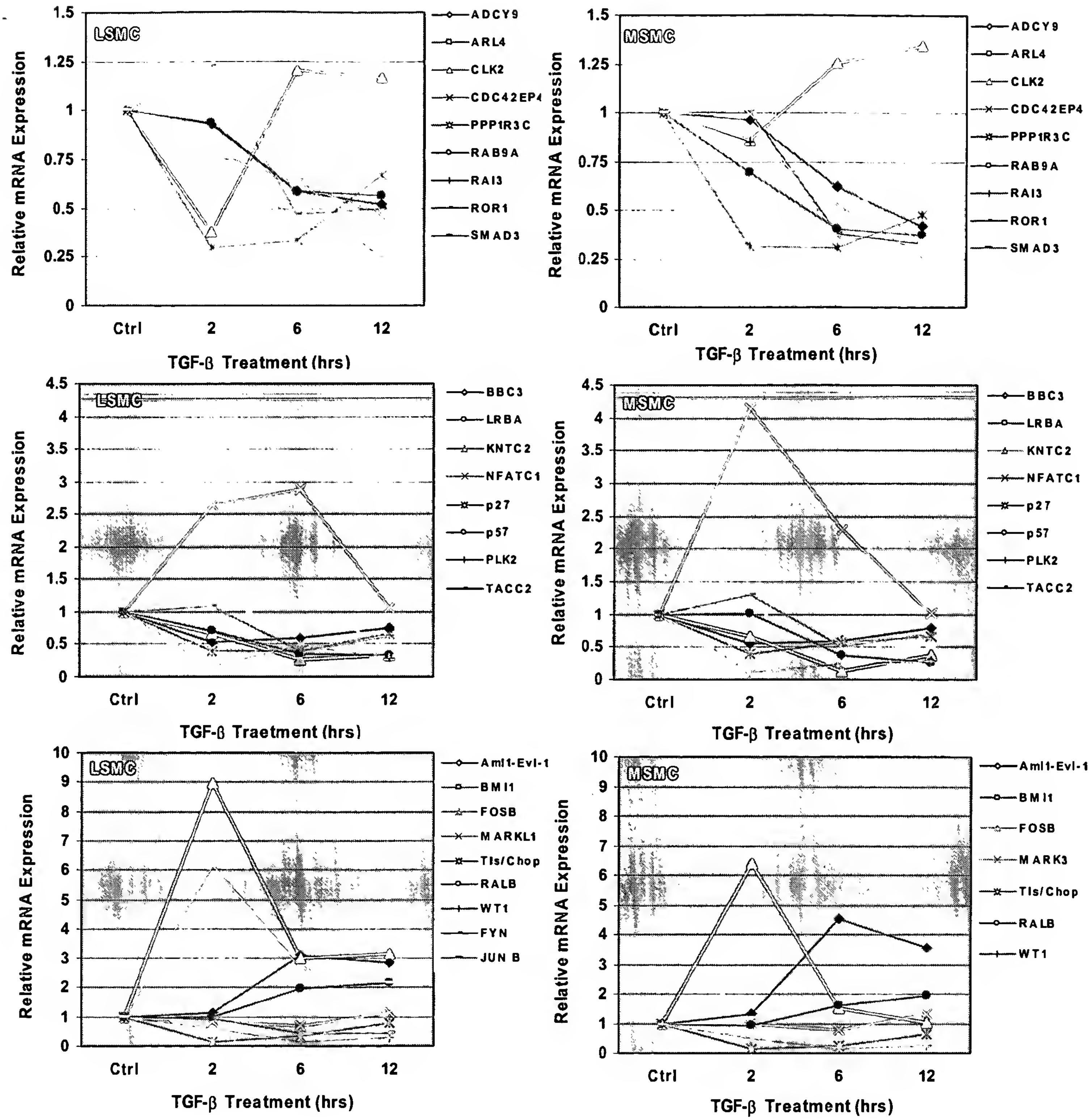
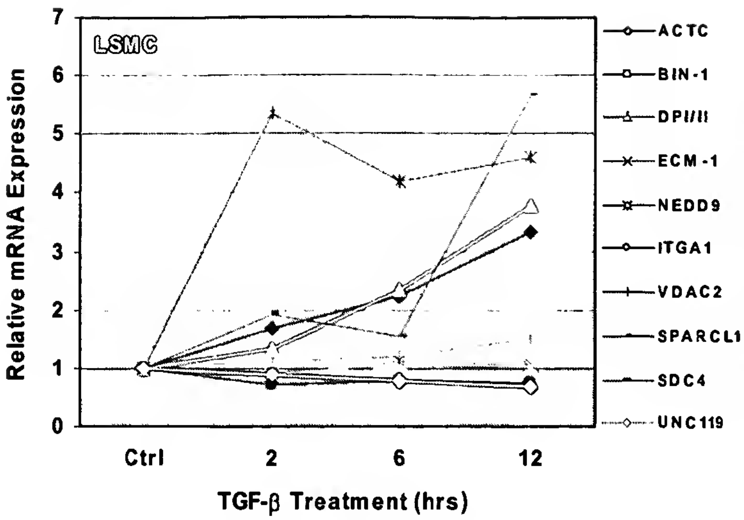


Figure 15 (Cont):



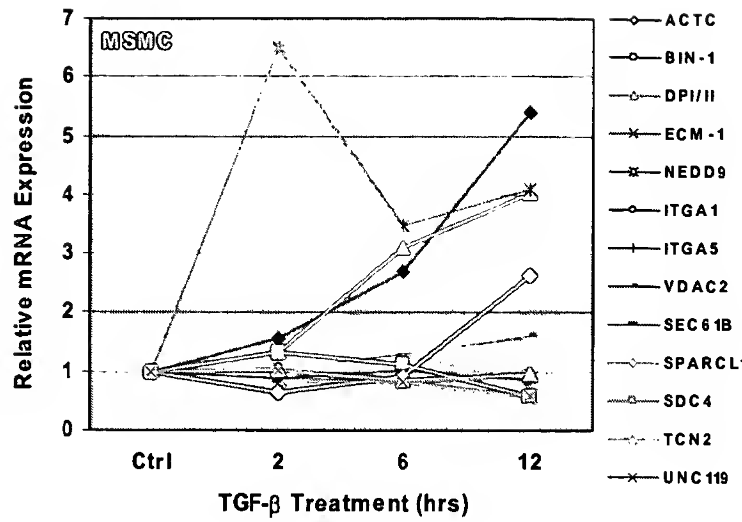


Figure 16:

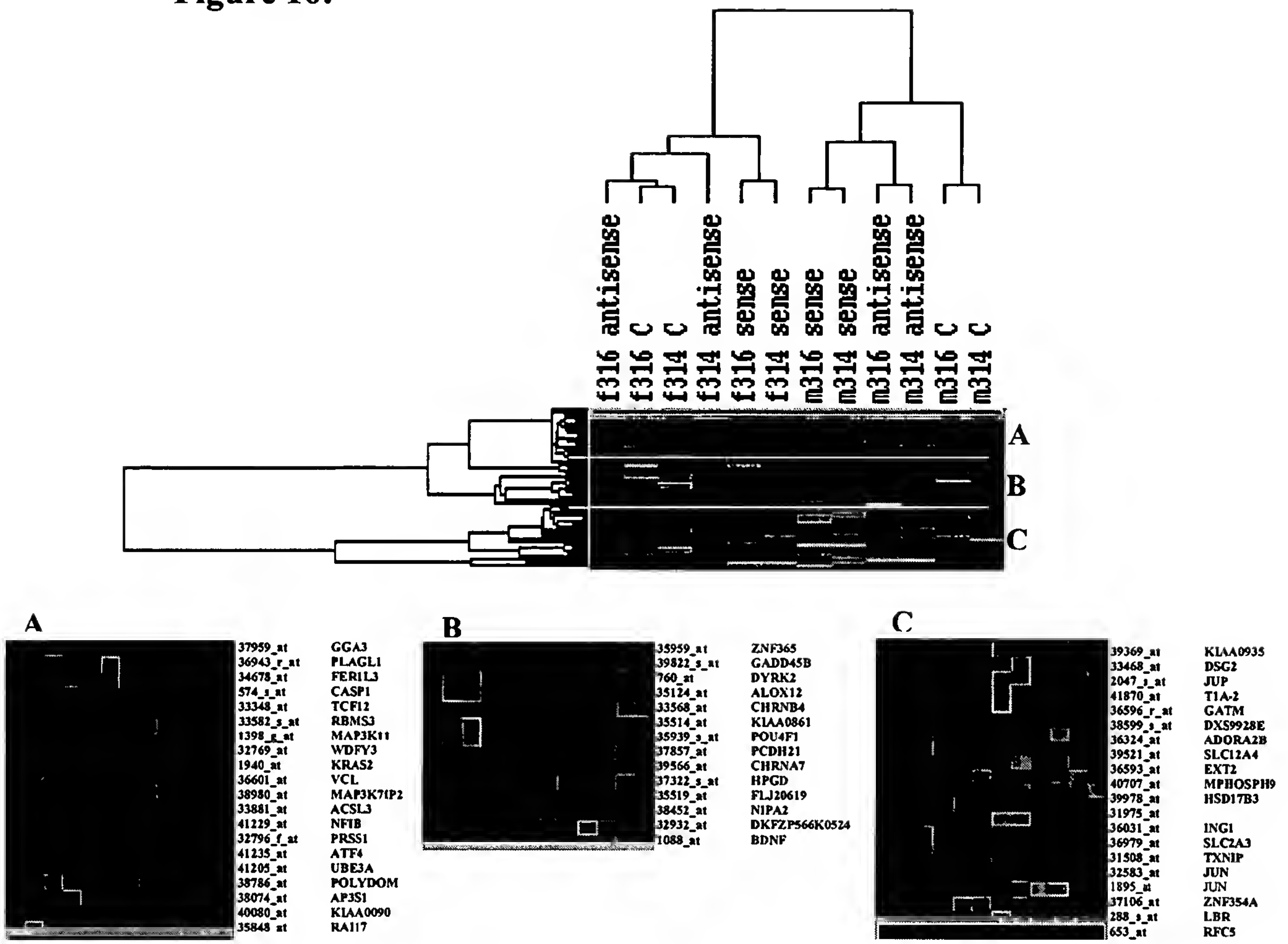

f316 antisense
f316 C
f314 C
f314 antisense
f316 sense
f314 sense
m316 sense
m314 sense
m316 antisense
m314 antisense
m316 C
m314 C
A
B
C
A
37959_at GGA3
36943_r_at PLAGL1
34678_at FER1L3
574_s_at CASP1
33348_at TCF12
33582_s_at RBMS3
1398_g_at MAP3K11
32769_at WDFY3
1940_at KRAS2
36601_at VCL
38980_at MAP3K7IP2
33881_at ACSL3
41229_at NFIB
32796_f_at PRSS1
41235_at ATF4
41205_at UBE3A
38786_at POLYDOM
38074_at AP3S1
40080_at KIAA0090
35848_at RAI17
B
35959_at ZNF365
39822_s_at GADD45B
760_at DYRK2
35124_at ALOX12
33568_at CHRNB4
35514_at KIAA0861
35939_s_at POU4F1
37857_at PCDH21
39566_at CHRNA7
37322_s_at HPGD
35519_at FLJ20619
38452_at NIPA2
32932_at DKFZP566K0524
1088_at BDNF
C
39369_at KIAA0935
33468_at DSG2
2047_s_at JUP
41870_at T1A-2
36596_r_at GATM
38599_s_at DXS9928E
36324_at ADORA2B
39521_at SLC12A4
36593_at EXT2
40707_at MPHOSPH9
39978_at HSD17B3
31975_at
36031_at ING1
36979_at SLC2A3
31508_at TXNIP
32583_at JUN
1895_at JUN
37106_at ZNF354A
288_s_at LBR
653_at RFC5

Figure 17:

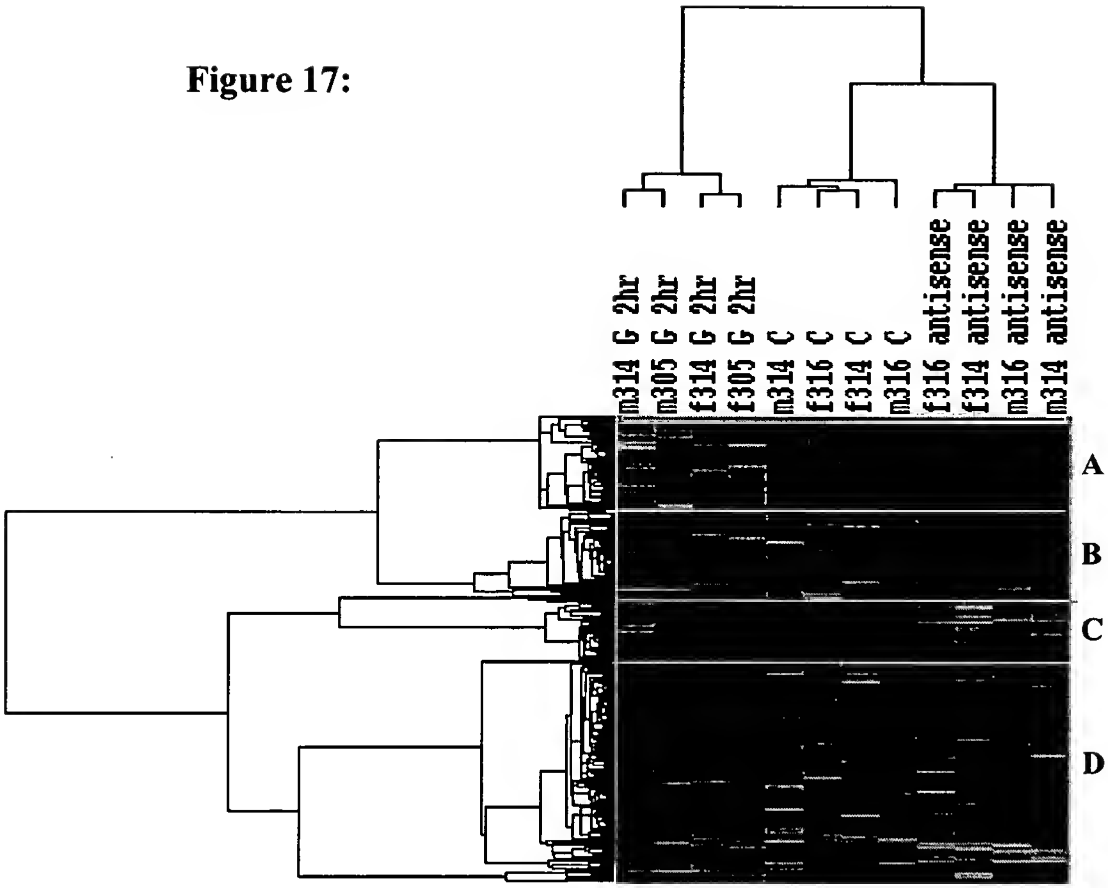
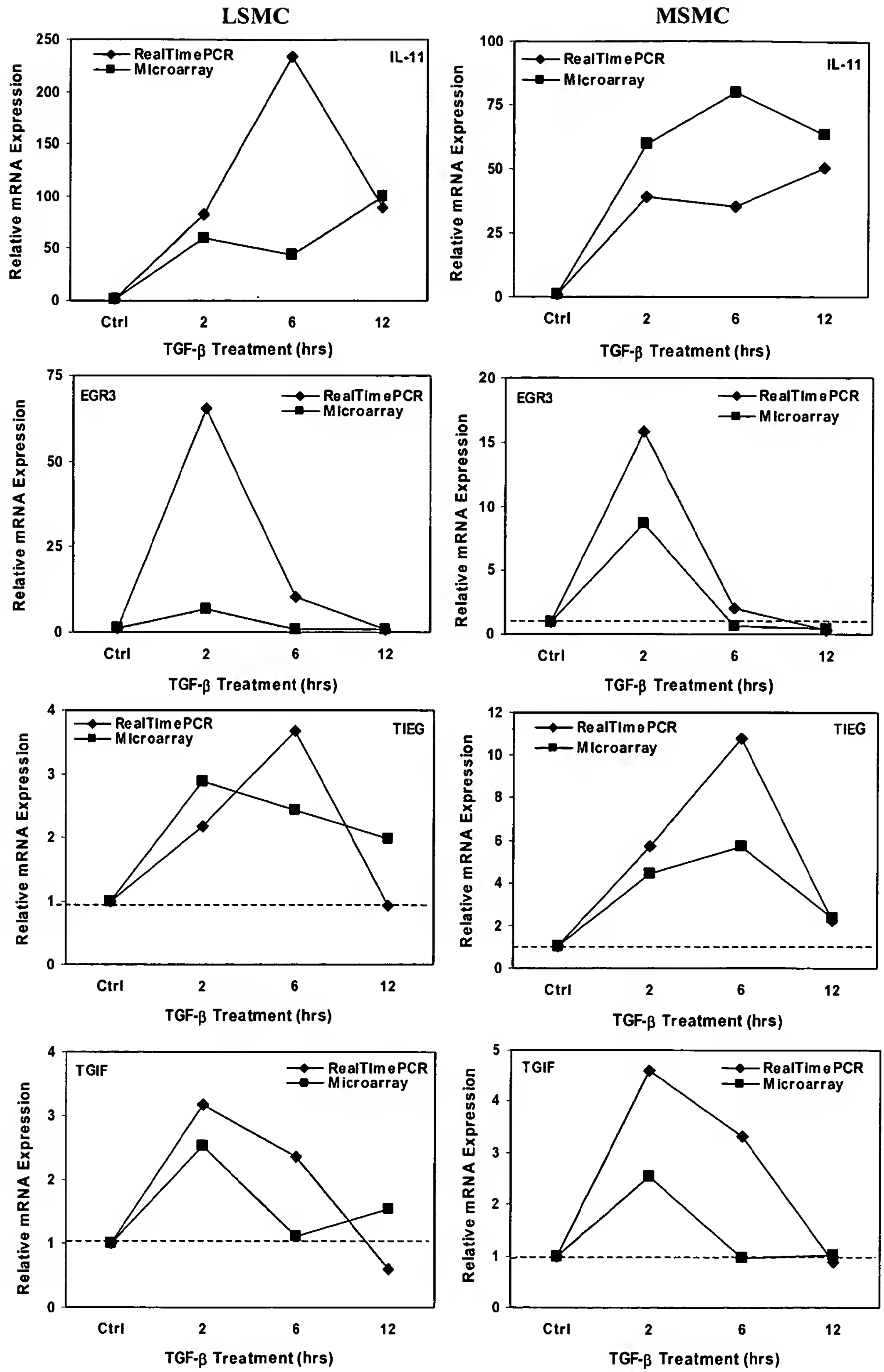

m314 G 2hr
m305 G 2hr
f314 G 2hr
f305 G 2hr
m314 C
f316 C
f314 C
m316 C
f316 antisense
f314 antisense
m316 antisense
m314 antisense
A
B
C
D

Figure 18:



LSMC
MSMC
IL-11
EGR3
TIEG
TGIF
RealTimePCR
Microarray
Relative mRNA Expression
TGF-β Treatment (hrs)
Ctrl
2
6
12

Figure 18 (Cont)

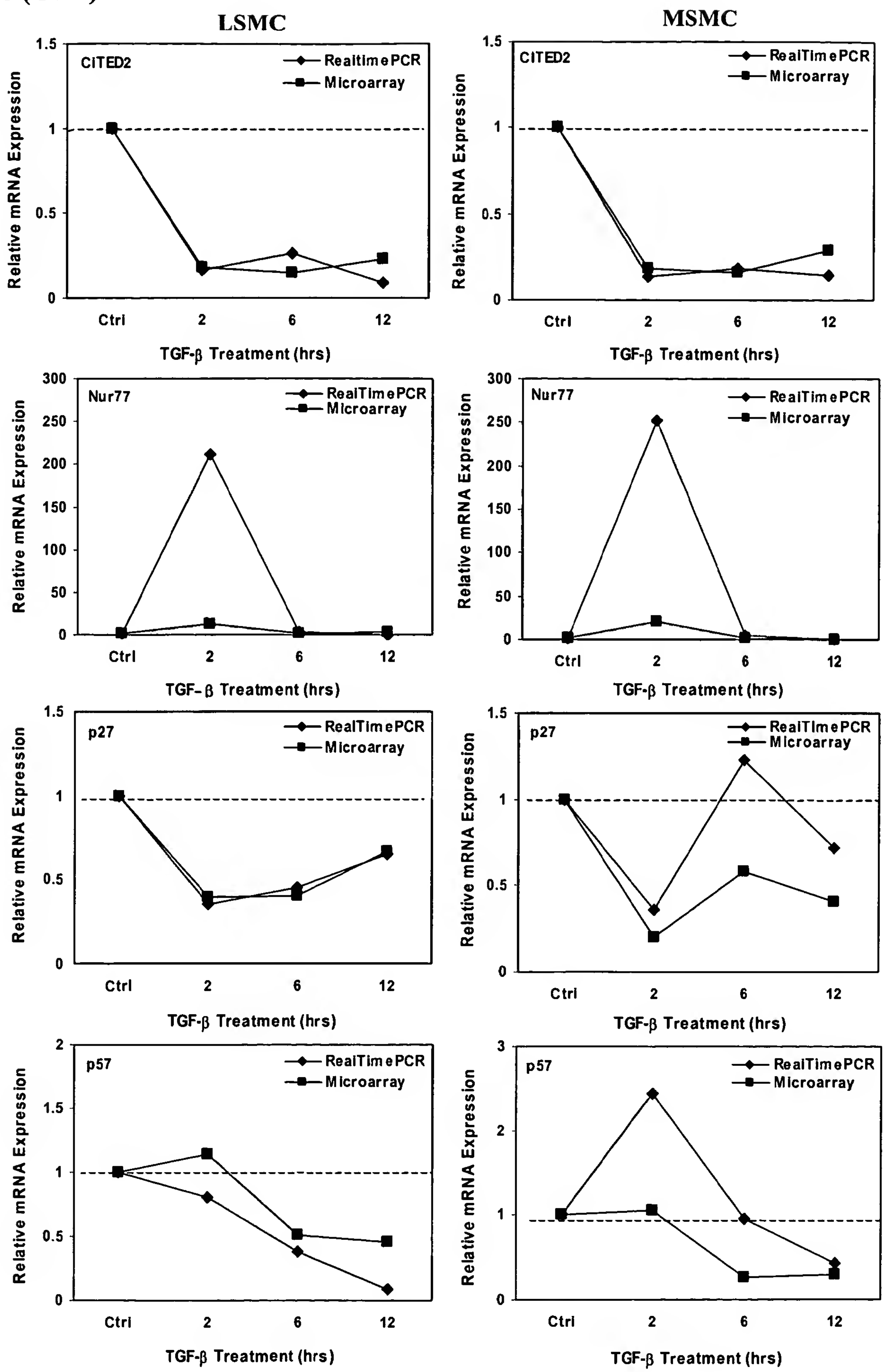
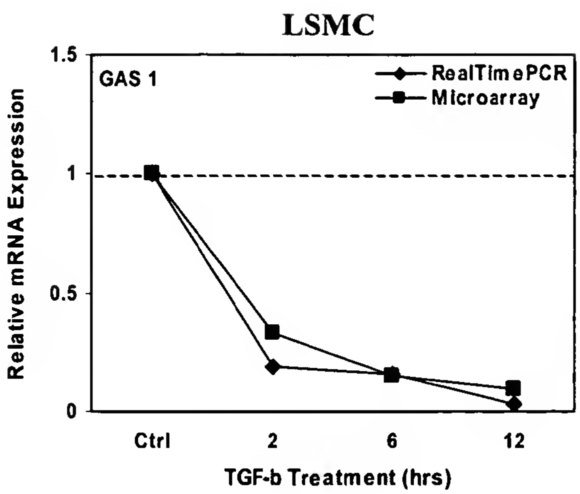


Figure 18 (Cont):



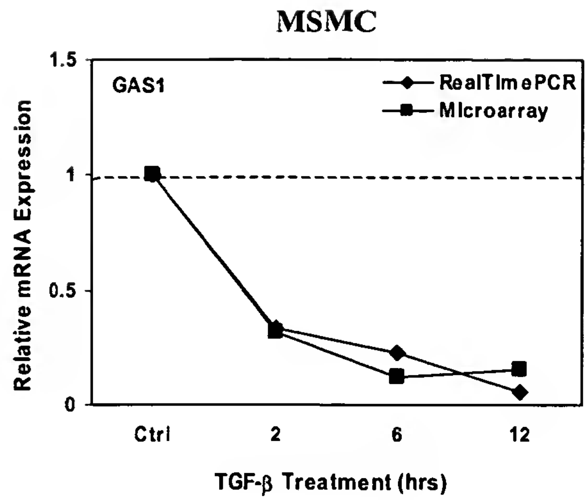
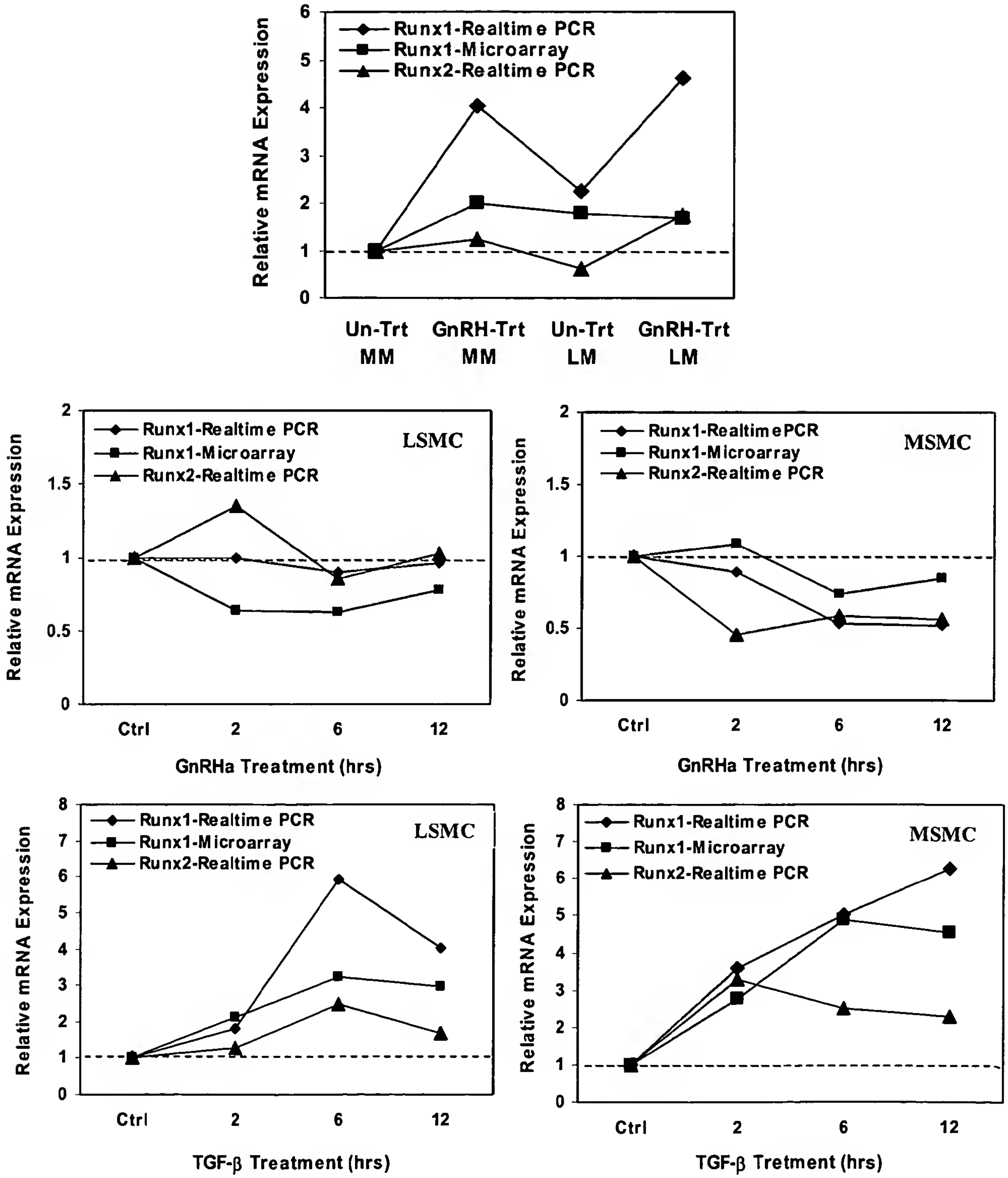


Figure 19:


Runx1-Realtime PCR
Runx1-Microarray
Runx2-Realtime PCR
Relative mRNA Expression
Un-Trt MM
GnRH-Trt MM
Un-Trt LM
GnRH-Trt LM
LSMC
GnRHa Treatment (hrs)
MSMC
GnRHa Treatment (hrs)
LSMC
TGF-β Treatment (hrs)
MSMC
TGF-β Tretment (hrs)
Ctrl
2
6
12

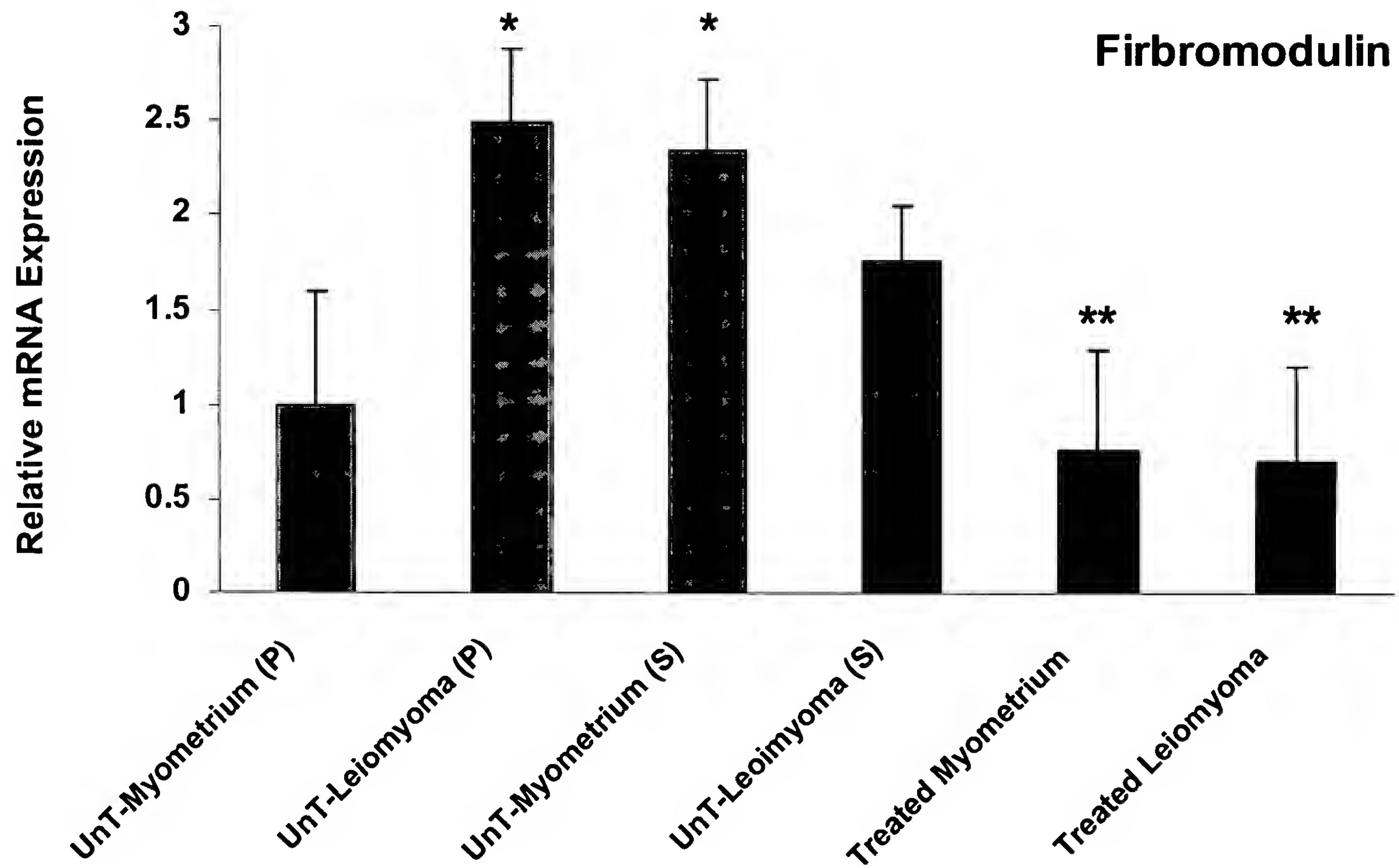

Firbromodulin
Relative mRNA Expression
3
2.5
2
1.5
1
0.5
0
*
*
**
**
UnT-Myometrium (P)
UnT-Leiomyoma (P)
UnT-Myometrium (S)
UnT-Leoimyoma (S)
Treated Myometrium
Treated Leiomyoma

FIG. 20

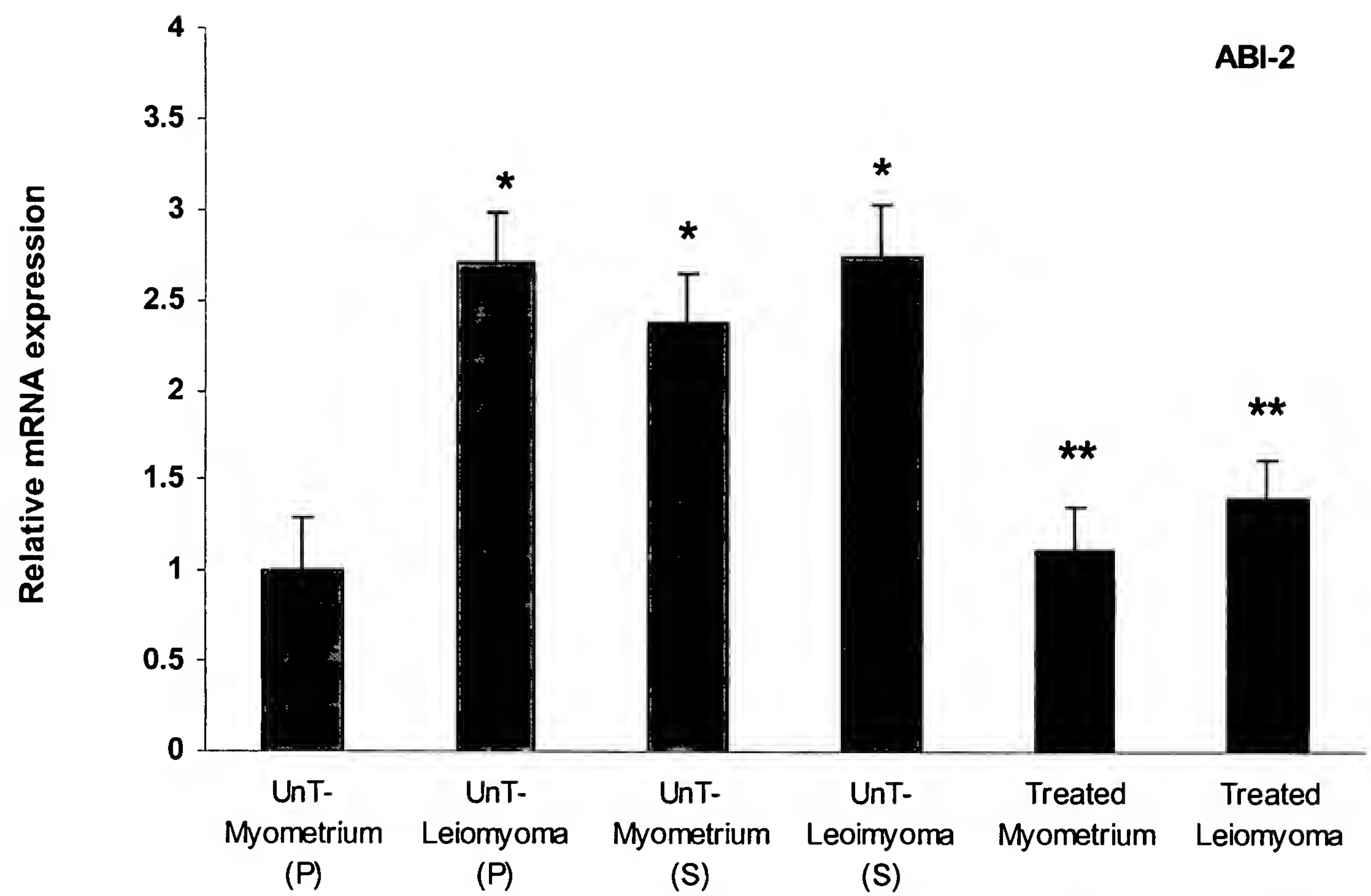

ABI-2
Relative mRNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
**
**
UnT-
Myometrium
(P)
UnT-
Leiomyoma
(P)
UnT-
Myometrium
(S)
UnT-
Leoimyoma
(S)
Treated
Myometrium
Treated
Leiomyoma

FIG. 21